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CONTENTS

No. 1, September 20, 1939

PAGE

Crozier, W. J., and Wolf, Ernst. The flicker response contour for the crayfish. I. 1

Hershey, A. D. Factors limiting bacterial growth. VI. Equations describing the early periods of increase 11

Chase, Aurin M., and Smith, Emil L. Regeneration of visual purple in solution 21

Jacques, A. G. The kinetics of penetration. XX. Effect of pH and of light on absorption in impaled *Halicystis* 41

Osterhout, W. J. V. Calculations of bioelectric potentials. V. Potentials in *Halicystis* 53

Northrop, John H. Increase in bacteriophage and gelatinase concentration in cultures of *Bacillus megatherium* 59

Hadidian, Zareh, and Hoagland, Hudson. Chemical pacemakers. Part I. Catalytic brain iron. Part II. Activation energies of chemical pacemakers 81

Crozier, W. J., and Holway, A. H. Theory and measurement of visual mechanisms. III. ΔI as a function of area, intensity, and wave length, for monocular and binocular stimulation 101

No. 2, November 20, 1939

Crozier, W. J., and Wolf, Ernst. Temperature and critical illumination for reaction to flickering light. V. *Xiphophorus*, *Platypoecilius*, and their hybrids 143

Wilkerson, Vernon A. The chemistry of human skin. IV. The electrokinetic effect of various ions upon suspended particles of stratum corneum 165

Osterhout, W. J. V. Calculations of bioelectric potentials. VI. Some effects of guaiacol on *Nitella* 171

Peters, Erich, and Saslow, George. Performance of the Hepp micro osmometer 177

Höber, Rudolf. Studies concerning the nature of the secretory activity of the isolated Ringer-perfused frog liver. I. The differential secretion of pairs of dyestuffs 185

PAGE

HÖBER, RUDOLF, and MOORE, ELINOR. Studies concerning the nature of the secretory activity of the isolated Ringer-perfused frog liver. II. The inhibitory and the promoting influence of organic electrolytes and non-electrolytes upon the secretion of dyestuffs 191

KALMANSON, G., and BRONFENBRENNER, J. Studies on the purification of bacteriophage 203

CROZIER, W. J., and WOLF, ERNST. The flicker response contour for the frog 229

ANSON, M. L. The denaturation of proteins by synthetic detergents and bile salts 239

ANSON, M. L. The reactions of denatured egg albumin with ferricyanide 247

TEORELL, TORSTEN. On the permeability of the stomach mucosa for acids and some other substances 263

NO. 3, JANUARY 20, 1940

WINNICK, THEODORE, DAVIS, ALVA R., and GREENBERG, DAVID M. Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. I. Chemical properties, activation-inhibition, pH-activity, and temperature-activity curves 275

WINNICK, THEODORE, DAVIS, ALVA R., and GREENBERG, DAVID M. Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. II. Kinetics of protein digestion by asclepain 289

WINNICK, THEODORE, DAVIS, ALVA R., and GREENBERG, DAVID M. Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. III. Kinetics of the heat inactivation of papain, bromelin, and asclepain 301

CROZIER, W. J. On the relation between birth weight and litter size, in mice 309

ANSON, M. L. The reactions of iodine and iodoacetamide with native egg albumin 321

THIMANN, KENNETH V., and COMMONER, BARRY. A differential volumeter for micro respiration measurements . . . . . 33[illegible]

GUTTMAN, RITA. Stabilization of spider crab nerve membranes by alkaline earths, as manifested in resting potential measurements. 343

OSTERHOUT, W. J. V., and MURRAY, J. W. Behavior of water in certain heterogeneous systems 365

PAGE

WHELDEN, ROY M., BUCHWALD, CHARLES E., COOPER, FRANKLIN S., and HASKINS, CARYL P. Electron bombardment of biological materials. II. The rate of death of fungus spores bombarded in vacuum with cathode ray beams from 4 kv.–15 kv. 391

No. 4, MARCH 20, 1940

CLOWES, G. H. A., and KRAHL, M. E. Studies on cell metabolism and cell division. III. Oxygen consumption and cell division of fertilized sea urchin eggs in the presence of respiratory inhibitors. 401

KRAHL, M. E., and CLOWES, G. H. A. Studies on cell metabolism and cell division. IV. Combined action of substituted phenols, cyanide, carbon monoxide, and other respiratory inhibitors on respiration and cell division 413

OSTERHOUT, W. J. V. Some chemical aspects of the potassium effect. 429

LI, CHOH HAO, LYONS, WILLIAM R., and EVANS, HERBERT M. Studies on pituitary lactogenic hormone. I. Electrophoretic behavior . . 433

HERRIOTT, ROGER M., DESREUX, VICTOR, and NORTHROP, JOHN H. Electrophoresis of pepsin 439

PRICE, JOHN W. Time-temperature relations in the incubation of the whitefish, *Coregonus clupeaformis* (Mitchill) 449

FRENCH, C. S. The pigment-protein compound in photosynthetic bacteria. I. The extraction and properties of photosynthin 469

FRENCH, C. S. The pigment-protein compound in photosynthetic bacteria. II. The absorption curves of photosynthin from several species of bacteria 483

BLINKS, L. R. Bioelectric potentials in *Halicystis*. VIII. The effects of light 495

No. 5, MAY 20, 1940

LYDDANE, R. H., and STUHLMAN, OTTO, JR. The theoretical response of living cells to contact with solid bodies 521

CROZIER, W. J., and WOLF, ERNST. Temperature and critical illumination for reaction to flickering light. VI. Flash duration varied . 531

BLINKS, L. R., and NIELSEN, JOHN P. The cell sap of *Hydrodictyon* . . 551

SUBBAROW, Y., and TRAGER, W. The chemical nature of growth factors required by mosquito larvae. II. Pantothenic acid and vitamin B_6 561

OSTERHOUT, W. J. V. Effects of hexylresorcinol on *Nitella* 569

COLE, WILLIAM H. The composition of fluids and sera of some marine animals and of the sea water in which they live 575

DOUDOROFF, MICHAEL. Experiments on the adaptation of *Escherichia coli* to sodium chloride 585

PAGE

McFarland, R. A., and Halperin, M. H. The relation between foveal visual acuity and illumination under reduced oxygen tension 613

Delbrück, M. Adsorption of bacteriophage under various physiological conditions of the host 631

Delbrück, M. The growth of bacteriophage and lysis of the host . . 643

No. 6, July 20, 1940

Inman, O. L. Studies on the chlorophylls and photosynthesis of thermal algae from Yellowstone National Park, California, and Nevada 661

Crozier, W. J., and Wolf, Ernst. Reaction to visual flicker in the newt *Triturus* 667

Crozier, W. J., and Wolf, Ernst. The flicker response curve for *Fundulus* 677

Anson, M. L. The purification of cathepsin 695

Krueger, A. P., Scribner, E. J., and Mecracken, T. The photodynamic inactivation of phage precursor by methylene blue 705

Hecht, Selig, and Pirenne, Maurice Henri. The sensibility of the nocturnal long eared owl in the spectrum 709

Loring, Hubert S. Solubility studies on purified tobacco mosaic virus 719

Barnes, T. Cunliffe. Summation of electrical potential of living membranes (frog skin): a model of the electrical organ of fishes . . 729

Li, Choh Hao, Evans, Herbert M., and Wonder, Donald H. Electrophoretic homogeneity of pregnant mare serum gonadotrophin 733

Jacques, A. G. Separation of potassium isotopes in *Valonia* and *Nitella* 741

Osterhout, W. J. V., and Hill, S. E. Action curves with single peaks in *Nitella* in relation to the movement of potassium 743

Osterhout, W. J. V. Effects of guaiacol and hexylresorcinol in the presence of barium and calcium 749

Velick, Sidney, and Gorin, Manuel. The electrical conductance of suspensions of ellipsoids and its relation to the study of avian erythrocytes 753

Ingram, M. The influence of sodium chloride and temperature on the endogenous respiration of *Bacillus cereus* 773

Index to Volume 23 781

THE FLICKER RESPONSE CONTOUR FOR THE CRAYFISH. I

By W. J. CROZIER and ERNST WOLF

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(Received for publication, March 1, 1939)

I

The flicker response contour for some typical arthropods exhibits a feature of particular interest. With *Anax*[1] (dragonfly, nymph) and *Apis*[2] (bee) the band describing log flash intensity (I) *vs.* flash frequency (F) for threshold reaction to flicker is pronouncedly asymmetrical. This has been attributed[3] to the statistically disadvantageous position of ommatidia at the sides of the markedly convex eye of these animals. An arthropod in which this limitation does not exist because the surface of the eye is sufficiently flat[4] gives a flicker response contour which is perfectly symmetrical. A symmetrical curve is also provided by the responses of vertebrates such as *Pseudemys*[5] (turtle) and gecko[6] where the excitatory elements involved constitute a single population. For these uncomplicated cases the flicker response contour ($F - \log I$) is properly described as a probability integral.[7] With most vertebrates the contour exhibits the duplex character associated with the possession of both retinal rods and cones; the asymmetry due to overlapping of the respective contributions to the $F - \log I$ curve is readily dealt with analytically.[8]

It is consistent with the mechanical interpretation of the $F - \log I$ asymmetry in arthropods with highly convex eyes that the curve is appropriately made more symmetrical by occluding all but the center of the optic surface.[9] It is also reduced by increasing the proportion of light time to dark time in the flash cycle;[10] the theory[11] here calls for a greater probability

[1] *J. Gen. Physiol.*, 1936–37, **20**, 363.
[2] *J. Gen. Physiol.*, 1935–36, **19**, 503; 1937–38, **21**, 223.
[3] *J. Gen. Physiol.*, 1936–37, **20**, 363; 1937–38, **21**, 223.
[4] *J. Gen. Physiol.*, 1938–39, **22**, 451.
[5] *Proc. Nat. Acad. Sc.*, 1938, **24**, 125, 216. *J. Gen. Physiol.*, 1938–39, **22**, 311.
[6] *Proc. Nat. Acad. Sc.*, 1938, **24**, 216. *J. Gen. Physiol.*, 1938–39, **22**, 555.
[7] *Proc. Nat. Acad. Sc.*, 1937, **23**, 71, 516. *J. Gen. Physiol.*, 1937–38, **21**, 313, *etc.*
[8] *J. Gen. Physiol.*, 1937–38, **21**, 203, 313, *etc.*
[9] *J. Gen. Physiol.*, 1937–38, **21**, 223.
[10] *J. Gen. Physiol.*, 1937–38, **21**, 463.
[11] *J. Gen. Physiol.*, 1937–38, **21**, 313.

of involving marginally illuminated elements which can be concerned in excitation, although at the same time requiring a reduction in the frequency with which they can contribute to the determination of the end-point result (*i.e.*, response), so that $F_{max.}$ is then decreased.[11, 10]

The crustacean *Uca* (fiddler crab)[12] and the nymph of *Aeschna*[13] (dragonfly) give additional evidence of the essential asymmetry of the curve of visual effect as a function of log I; the conditions of the experiments in these cases were such as probably to increase the effect. Similar indications are contained in the data on *Drosophila*,[14] and in observations on the visual acuity and intensity discrimination of the bee.[15]

The asymmetry of the flicker response contour for typical large-eyed arthropods, and of the corresponding curves for visual acuity, is thus seen to be modifiable according to differences in the technic of utilizing responses to a moved system of opaque and illuminated stripes, by changes in the area of optic surface open to illumination, and by alteration of the light time fraction in the light cycle. It does not change as a function of temperature.[16] The modification of asymmetry is in each instance consistent with the requirements of the view that the $F - \log I$ contour is fundamentally a symmetrical probability integral, in typical arthropods distorted by the fact that normally the intensity required for response is, at lower flash frequencies, unable to act upon the full complement of potentially excitable elements because they are not adequately oriented to receive sufficient light; the higher intensities required for higher levels of F in effect increase the usable area of the retina. The formal completion of this general argument is provided by the fact that neither change of area, light time ratio,[11] nor temperature[17] alters the symmetry of the "rod" or "cone" curves for vertebrates.

To test the strength of this interpretation has seemed desirable, since for a general theory of visual responses such as may depend upon probability considerations a valid explanation must be provided for the exceptional

[12] *J. Gen. Physiol.*, 1937–38, **21**, 223 (p. 239; data in Clark, L. B., *J. Gen. Physiol.*, 1935–36, **19**, 311).

[13] *J. Gen. Physiol.*, 1937–38, **21**, 463 (p. 471; data in Sälzle, K., *Z. vergleich. Physiol.*, 1932, **18**, 347).

[14] Data in Hecht, S., and Wald, G., *J. Gen. Physiol.*, 1933–34, **17**, 517.

[15] Hecht, S., and Wolf, E., *J. Gen. Physiol.*, 1928–29, **12**, 727. Wolf, E., *J. Gen. Physiol.*, 1932–33, **16**, 407, 773. Wolf, E., and Crozier, W. J., *J. Gen. Physiol.*, 1932–33, **16**, 787; 1935–36, **19**, 503; 1937–38, **21**, 223, 463.

[16] *J. Gen. Physiol.*, 1936–37, **20**, 393.

[17] *J. Gen. Physiol.*, 1936–37, **20**, 411. *Proc. Nat. Acad. Sc.*, 1938, **24**, 216. *J. Gen. Physiol.*, 1938–39, **22**, 795.

cases not adhering to the probability integral. Moreover, the interpretation of the visual performance curves for arthropods requires a usable interpretation of their peculiar form as contrasted with those for vertebrates. In making such a further test two points have been especially in mind:

1. It is implied that an arthropod with eye-surfaces more convex than those thus far used should exhibit in its $F - \log I$ curve a more pronounced asymmetry. By suitable reduction of the optic area it should be possible to reduce the asymmetry; an experiment of this sort is described in a following paper.

2. For *Apis*, *Anax*, and *Asellus* the standard deviation of $dF/d \log I$, with $F_{max.} = 100$ per cent, denoted by $\sigma'_{\log I}$ is so far as can be judged identical.[4] This, of course, refers to the upper part of the curve, beyond the intensity at which the asymmetrical "discrepancy" vanishes. Reasons already listed have shown why this function is taken to be the underlying, fundamental description of the cumulative frequency distribution of elemental excitabilities. For vertebrates this quantity is a characteristic invariant differing markedly from one kind of animal to another[5, 6, 8] and apparently determined specifically by the relevant genetic constitution of the animal.[18] For both *Anax*[16] and vertebrates[17] $\sigma'_{\log I}$ is independent of temperature and of fractional light time in the flash cycle,[10, 11] and for *Anax*[9] (and human also) it is independent of retinal area, it is clearly an invariant of the type which rational physiological analysis requires and must seek to be in position to invoke. The quantitative agreement in $\sigma'_{\log I}$ for *Anax*, *Apis*, and *Asellus* (unlooked for and unsuspected when the experiments were made) is so close as to signify a common basis in the physical organization of these arthropods. In view of other pertinent evidence this is a remarkable state of affairs, which seems unlikely to be accidental.[4] It could be true that arthropods with still more highly convex eyes might show a different value of $\sigma'_{\log I}$, particularly if the eyes are carried on independently moving stalks.

II

A form convenient for these tests is the crayfish *Cambarus bartoni*. The eyes are here more convex than in *Anax*, for example. With these animals several further types of experiment are possible. For the stock involved the data of Table I may be taken as a norm, with which (under control by check observations) the results of certain experimental treatments will subsequently be compared.

[18] (In man there is some evidence to suggest that it may change slightly but systematically with age also.)

Individuals 2.5 to 4 cm. long were selected from a large number being raised in the laboratory. Larger individuals tend to be less consistently active. They were kept in separate dishes and fed regularly with dried shrimp and *Elodea*. The same 10 crayfish, with identifying numbers, were used throughout. The technic of preparation and observation was the same as that recorded in our earlier papers. After dark adaptation for several hours at 21.5°C., threshold reaction to visual flicker was observed at this temperature, employing measurements of critical illumination in the flash at various fixed flash frequencies. The flash cycle used had equally long light and dark intervals. With slowly increasing intensity, the first definite progressive motion is taken as index of the desired end-point; movement of the antennae is too uncertain for this purpose. The reaction consists in walking movements in the direction of motion of the striped cylinder.[19] This occurs very quickly when the threshold intensity has been reached. If the crayfish is at the center of its jar movements of the antennae occur first, then flexion and extension of the claws, and finally progression. The sequence of these three movements is rapid, so that with an active individual they can come at apparently the same time. If the *Cambarus* is at the wall of its jar and facing in the direction of the stripe motion, walking begins once threshold I is reached; if facing in the opposite direction, movements of antennae and legs are the first signs of reaction, tending to push the animal backward. In some cases a crayfish in the latter position tends to turn around, toward the wall of the jar. A *Cambarus* oriented radially in the jar, with the head end against the wall, will occasionally show first of all a response involving merely movements of the claws, followed by turning and creeping in the direction of motion of the stripes. The measurements of critical intensity for response were chiefly based upon reactions of animals not close to the wall of the containing jar, but in fact no real differences are found when using the other modes of response described.

In view of the diurnal cycles of retinal activity found in crustaceans,[20] involving movements of retinal pigment, it is to be noted that readings were taken, at several flash frequencies, during mid-afternoon hours only.

The observations are collected in Table I. The 10 crayfish used form a homogeneous[19] lot, in the sense that the relative sensitivity of each individual is randomly distributed in time and shows no correlation in successive tests on the same day; the difference between extreme mean rank order numbers of 2 individuals $= 3.1 \times \text{P. E.}_{diff.}$. The dispersions of the critical intensities are of the same order as found in *Asellus*,[4] but are a little smaller. The scatter of the determinations of critical intensity is directly proportional to the mean (Fig. 1).

III

The $F - \log I_m$ curve for *Cambarus* is markedly asymmetric (Fig. 2). The *relative* distortion of the curve is greater than with bee and dragonfly

[19] *J. Gen. Physiol.*, 1936–37, **20**, 211, 363–393; 1937–38, **21**, 17, *etc.*

[20] Welsh, J. H., *Proc. Nat. Acad. Sc.*, 1930, **16**, 386. *Biol. Bull.*, 1936, **70**, 217. Under conditions of *continued* darkness diurnal migrations of retinal pigment occur in *Cambarus* also, as Dr. Welsh has kindly informed us on the basis of his recent work.

nymph (Fig. 3). This was expected (section I) on the basis of the greater curvature of the retinal surface of the crayfish.

On a probability grid (Fig. 3) the sharp breaking of the curve at log I_m = *ca.* $\bar{1}.1$ separates the function into two parts: below this level there is wide departure from the course of the curve fitting the upper portion of the data. The departure is of exactly the same kind as with bee and dragonfly larva. The agreement in the general form of the distorted part of the curve

TABLE I

Mean critical flash intensities, with the probable errors of the dispersions, as log I_m and log P.E.$_{1I_1}$, millilamberts for various flash frequencies (F per sec.), for *Cambarus bartoni*. Flash cycle with equally long dark and light period, *i.e.* $t_L/t_D = 1$; temperature = 21.5°. At each F, three observations on each of the same 10 individuals used at all points. The individuals are shown to be an essentially homogeneous group (*see text*).

F	log I_m	log P.E.$_{1I_1}$
2	$\bar{4}.4955$	$\bar{5}.0785$
3	$\bar{3}.0976$	$\bar{5}.2420$
5	$\bar{3}.6017$	$\bar{5}.6766$
7	$\bar{3}.8898$	$\bar{4}.3462$
10	$\bar{2}.1483$	$\bar{4}.5181$
15	$\bar{2}.3993$	$\bar{3}.0302$
	$\bar{2}.3558$	$\bar{4}.3508$
20	$\bar{2}.5851$	$\bar{4}.9976$
	$\bar{2}.5816$	$\bar{4}.8320$
25	$\bar{2}.7744$	$\bar{3}.2862$
30	$\bar{2}.9442$	$\bar{4}.9294$
35	$\bar{1}.1014$	$\bar{3}.1486$
40	$\bar{1}.3564$	$\bar{3}.8599$
43	$\bar{1}.6424$	$\bar{3}.9375$
45	$\bar{1}.9352$	$\bar{2}.2167$
47	0.2997	$\bar{2}.7560$
48	0.5280	$\bar{1}.0103$
49	0.8756	$\bar{1}.0178$
50	1.6442	0.2579

(Fig. 4) can scarcely be fortuitous. It finds a rational explanation in the increasing probability of the involvement of marginal ommatidia as the critical intensity is increased through elevation of F; the disadvantage of the marginal ommatidia is due to the mechanical conditions affecting their reception of light, and not to an intrinsically lower excitability.[9]

The slope of the fundamental curve (Fig. 3) is measured by $\sigma'_{\log I}$, the standard deviation of the frequency distribution $dF/d \log I$ *vs.* log I (with

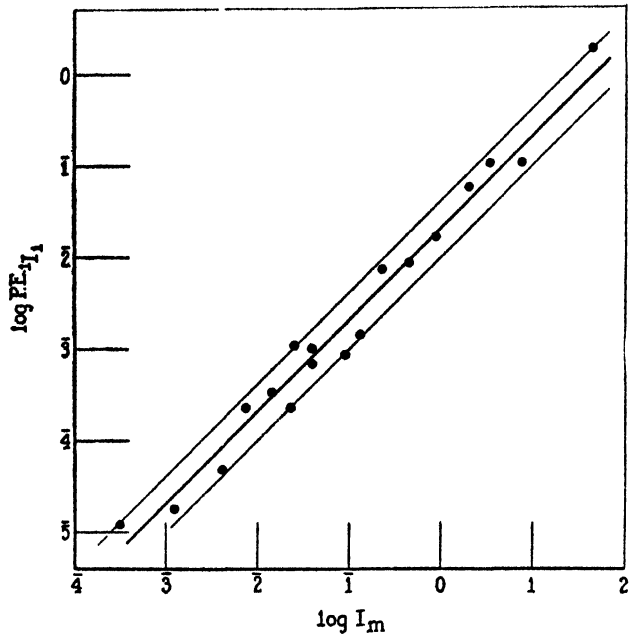

FIG. 1. The dispersion (P.E.$_{I_1}$) of the determinations of critical intensity for reaction of *Cambarus* to visual flicker is directly proportional to the mean critical intensity. Data in Table I.

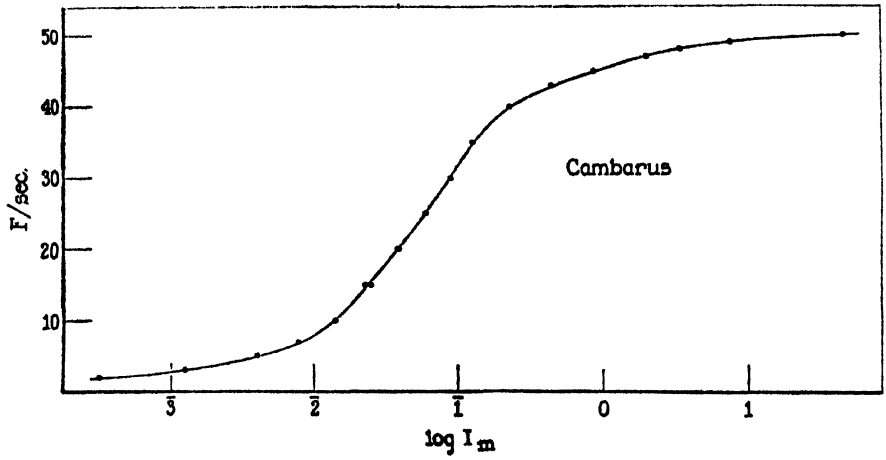

FIG. 2. The sigmoid relation between log I_m and F for *Cambarus* (Table I). The curve is not symmetrical (see Fig. 3).

$F_{max.}$ = 100 per cent). The fact has been commented upon[4] that this quantity is the same for such different animals as bee, dragonfly nymph, and isopod. For *Cambarus* it is clearly much greater (Fig. 3). This signifies that the frequency distribution for the excitability thresholds of the neural elements concerned is more widely spread. It may be entertained as a suggestive possibility that the great curvature of the optic surface, perhaps combined with the position of the eyes upon independently movable stalks as contrasted with the condition in *Anax*, *Asellus*, *Apis*, contributes in a

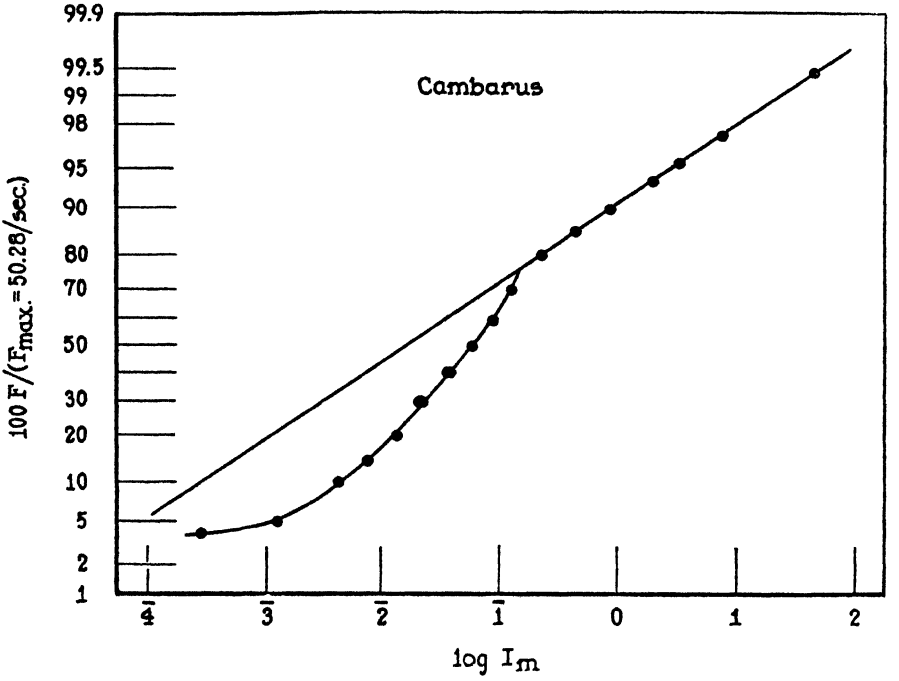

FIG. 3. The F − log I_m data for *Cambarus* (Fig. 2) shown upon a probability grid. The departure below *ca.* 74 per cent of $F_{max.}$ is discussed in the text.

decisive way to a statistically greater spread of the curve by increasing the possibility of moment-to-moment fluctuation in the effective thresholds of individual ommatidia.

These considerations apply to $\sigma'_{\log I}$, and for different animals it is to be noted that there is no correlation to be found between the absolute values of $F_{max.}$ and other properties of the flicker response contour. For *Anax*, under the same conditions of observation, $F_{max.}$ = 60.9, for *Cambarus*, 50.3; yet the "break" in the F − log I curve comes at precisely the same

level of intensity and of 100 $F/F_{max.}$ (Fig. 3); for *Apis* (by a slightly different method of observation)[2] the break comes at precisely the same *relative* level of F (Fig. 3).

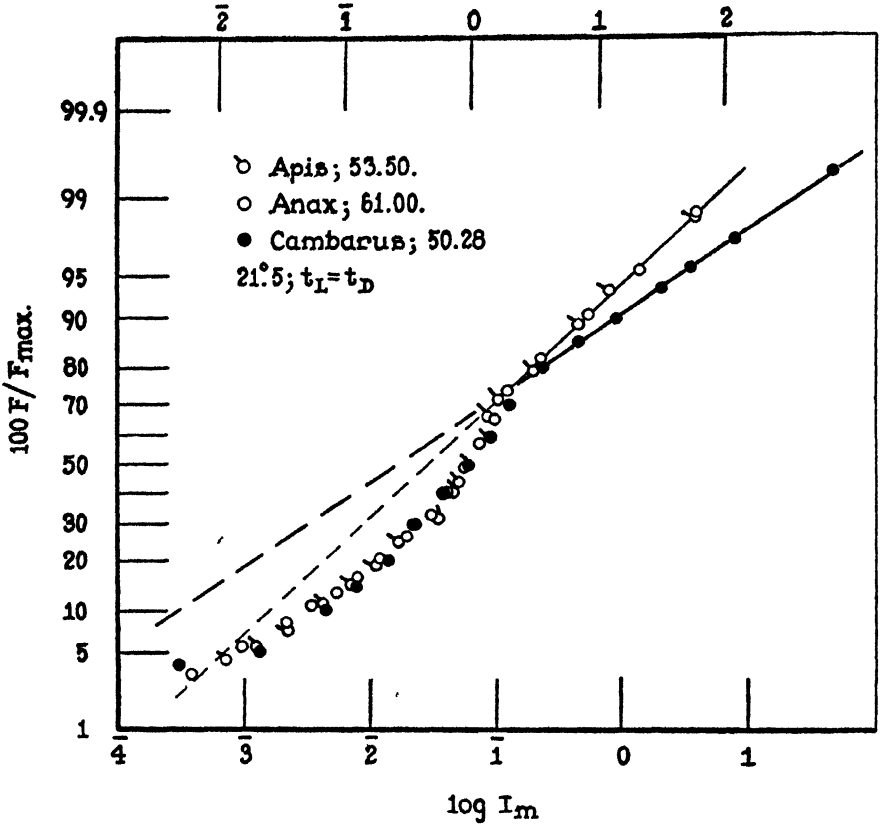

FIG. 4. The slope of the probability integral expressing the "fundamental curve" for *Cambarus* is less than for *Apis* and *Anax* and *Asellus*; the form of the curve for *Anax* and *Apis* is essentially the same, but separated by 1.22 units on the log I axis; this slope is identical with that obtained for the uncomplicated curve given by the comparatively flat-eyed *Asellus*.[4] The shape of the discrepant parts of the three curves, discussed in the text, is very nearly the same.

In terms of the analysis we are engaged upon, $F_{max.}$ is to be regarded as a measure of the total number of neural effect units capable of involvement in the discrimination of flicker.[21] The curious fact that (with $t_L/t_D = 1$) $F_{max.}$ is for a great variety of forms (dark adapted) confined to a compara-

[21] *J. Gen. Physiol.*, 1937–38, **21**, 17.

tively very narrow range of values (= 48 to 63) has been commented on;[22] however, in one animal (gecko[6]) this parameter is as low as 27 per second. The properties of the curves for different forms can be compared, however, only after reduction to a percentage scale ($F_{max.}$ = 100). For sundry convex-eyed arthropods upon which data are available—*Apis*,[9, 15] *Anax*,[9] *Uca*,[9] *Drosophila*[14]—as well as for the present data on *Cambarus*, the rate of increase of the slope $dF/d \log I$ increases up to F = *ca.* 70–74 per cent of $F_{max.}$ (*cf.* Fig. 3). This fact of itself suggests that a common physical factor is responsible for the peculiar form of the curves. The deduction is reinforced by the consideration that (Fig. 4) the form of the "discrepant" portion of the $F - \log I$ curve, responsible for the asymmetry of the contour as a whole, is remarkably similar. If the common physical factor is taken to be the convexity of the optic surface (and consequent angular divergence of the ommatidial axes) the congruence of the (non-specific) forms of the "discrepant" portions of the several curves in Fig. 4 is readily accounted for. The form of the $F - \log I$ curve in the region of lower critical intensities is thus explained by the fact that, F being made greater, a higher critical I is necessitated, which makes possible the involvement of a larger number of receptor units—the effective area of the eye is therefore increased—and the relative rate of increase of critical intensity per unit increase of F is therefore less than if the factor of ommatidial angular divergence were not involved.

The position can be further checked by investigating the effect of internally isolating the ommatidia by utilizing the migrations of retinal pigment induced by hormones acting upon crustacean chromatophores, as discussed in a succeeding paper.[23]

The final fact to be recognized is that in these data the value of $\sigma'_{\log I}$ for the "fundamental curve" (*cf.* Fig. 3) is definitely greater than for *Apis* and *Anax*, and than that for *Asellus* (no asymmetry). The statistical conception of the involvement of excitation elements in the determination of response rather distinctly calls for this finding in the present case. The optic surfaces of *Cambarus* are not only more convex, but the two eyes are independently movable. A wider variety of chances is thereby presented for the photic excitation of the various peripheral units. This implies a higher value of $\sigma'_{\log I}$, such as Fig. 3 demonstrates. The conclusion can be checked by measurements with other crustaceans.[24]

[22] *J. Gen. Physiol.*, 1936–37, **20**, 393.

[23] Crozier, W. J., and Wolf, E., 1939, *Biol. Bull.*, **77**, 126.

[24] The visual acuity data with the fiddler crab *Uca* (Clark, L. B., *J. Gen. Physiol.*, 1935–36, **19**, 311) were obtained by means of tests with a *single* moving stripe. This

We are indebted to Dr. Gertrud Zerrahn-Wolf for assistance in connection with the observations.

IV

SUMMARY

The $F - \log I$ curve for threshold response to visual flicker has been determined for the crayfish *Cambarus bartoni*. As predicted on the basis of the higher curvature of the optic surface, the flicker response contour is more asymmetrical than for bee and dragonfly nymph under comparable conditions of temperature and light time fraction of flash cycle. The mechanical origin of this asymmetry is thus confirmed, and is further supported by the similar forms of the $F - \log I$ curves in bee, dragonfly larva, and crayfish in the lower portion of the curves (up to $F = 70$ per cent $F_{max.}$). The slope of the fundamental curve for crayfish, deduced by analysis of the data, is lower than for bee, dragonfly nymph, or *Asellus*. This signifies a wider spread of the effective distribution of elemental log I thresholds involvable in the response to flicker, and may be traced either to the greater curvature of the eye-surfaces or to their position upon movable pedicles. The results are therefore consistent with the statistical conception of the nature of effects recognizable as due to the activity of excitable elements.

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inevitably implies, from the standpoint herein used, a *steeper*[12] log I curve—since a smaller number of excitation units will be produced by movement of a single stripe than by the use of a series of them. The importance of the number of light-dark transitions has been commented upon in other connections.[15] Similarly, in tests of visual acuity[15, 21] the striped plates are moved *slowly*. This again implies a low number of light-dark excitations as compared with the situation in tests for recognition of (response to) flicker. A smaller number of excitation units implies lesser relative variability, and consequently a smaller value of $\sigma'_{\log I}$, as is found for the bee.[2] The decisive comparisons of such data require further experiments with forms such as *Uca*, which we expect to make shortly.

FACTORS LIMITING BACTERIAL GROWTH

VI. Equations Describing the Early Periods of Increase

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By experiments involving direct measurements of size and growth rate of *Bacterium coli* in nutrient broth (1–3), evidence has been obtained for the following assumptions regarding bacterial growth:

1. Measured as increase of bacterial substance, growth occurs at constant rate under conditions we wish to consider.[1]

2. There is a latent period (L) during which the average cell undergoes no multiplication, but steadily increases in size to a maximum, the maximal average adult size (S_m). This size is characteristic of the species, but is affected by influences similar to those affecting the rate of growth.

3. At the end of the latent period, the average cell abruptly divides into two equal daughter cells, which in turn increase in size to the same maximal value before undergoing fission to produce the second generation. After the latent period is passed, therefore, the size changes $S_m/2 \rightleftarrows S_m$ prevail, and the rate of multiplication is numerically equal to the rate of accretion.

4. The individuals of a culture do not behave exactly as the average cell behaves (chiefly because of differences in initial size), but exhibit properties distributed symmetrically about the mean.[2] Thus the first division of the average cell is preceded and followed by an equal number of first divisions of other cells, fewer in number as they occur further in advance or arrears of the mean. As a result the rhythm of divisions of the majority is soon masked by the divisions of precocious and backward cells. Since the culture now contains cells distributed at random in all stages of fission, the average observed size (Sa) will be three-fourths of the average

[1] For *Bacterium coli*, nutrient broth cultures containing 1 to approximately 10^8 organisms per ml., with carbon dioxide available, and oxygen in excess.

[2] Or, not improbably, skewed. But the accuracy of the data to be considered does not warrant making this distinction.

maximal adult size, until altered by changing environmental circumstances.

Only the first of these assumptions is entirely new. Regarding the third, the evidence is contradictory. Jensen (4) noted that once a given cell had divided, it continued to do so for a time at a constant rate. Others (5, 6), on the contrary, have observed the successive shortening of generation time during early cell divisions which the concept of lag (if applicable to a single cell) implies. However, microscopic observations are almost necessarily limited to cultures on solid mediums, where significant changes in environmental conditions conceivably take place from generation to generation. If only for this reason, it appears justifiable to consider by themselves the results obtained with the present methods.

In what follows we are seeking more rigorous quantitative test of the assumptions outlined above, than was provided by the underlying experiments.

During the latent period, if the number of cells be considered constant (B_0), the increase in average size from S_0 to S_a occurs at constant rate. Then:

$$\frac{dS}{dt} = KS$$

Integrating between the limits S_0 and S_a,

$$t_L = \frac{1}{K} \ln \frac{S_a}{S_0}, \tag{1}$$

where

t_L = net time in hours occupied by growth in size.
K = fractional increase in size per hour.
S_a = average adult size.
S_0 = average initial size.

After all the cells have divided, the average size S_a remains constant, and the cell numbers increase at constant rate K. Therefore:

$$\frac{dB}{dt} = KB$$

And on integration,

$$t_n = \frac{1}{K} \ln \frac{B}{B_0}, \tag{2}$$

where

t_n = net time occupied by increase in numbers.
K = fractional increase in numbers per hour.
B = final numbers of cells.
B_0 = initial numbers.

But

$$t_L + t_n = \text{total elapsed time } t.$$

Substituting (1) and (2) into the above:

$$\frac{1}{K} \ln \frac{S_a}{S_0} + \frac{1}{K} \ln \frac{B}{B_0} = t,$$

and

$$\ln B = \ln B_0 + K t - \ln \frac{S_a}{S_0}.$$

But,

$$K = M \ln 2.^3$$

Then

$$\log B = \log B_0 + M t \log 2 - \log S_a/S_0. \tag{3}$$

Equation (3) predicts bacterial numbers expected at any time after the end of the latent period. Under given conditions of cultivation, M and S_a are constant and B varies only with B_0, t, and S_0.

The value of t_L (equation 1) is not measurable experimentally. But an expression for the latent period (L) representing the time required for growth in size from S_0 to S_m, is derived in a similar way. Thus:

$$L = \frac{1}{K} \ln \frac{S_m}{S_0}$$

Since, from postulate (4), $S_m = 4/3\ S_a$:

$$L = \frac{1}{M \log 2} \log \frac{4S_a}{3S_0} \tag{4}$$

---

3 $$M = \frac{K}{2.3 \log 2} = \frac{1}{t} \log \frac{B}{B_0}$$

where M is the reciprocal of the generation time as given by the equation of Buchner (7). M is defined, accordingly, as doublings per hour, or as generations per hour where growth proceeds by binary fission.

Equation (4) refers to the latent period of the average individual cell. For experimental purposes, therefore, L may be defined as the time required for 50 per cent increase in numbers, if it be assumed that the distribution of times of first division of individual cells is normal. Under given conditions of cultivation, L should vary only with S_0.

TABLE I

Observed and Calculated Latent Period

$$\text{Equation (4): } L = \frac{1}{M \log 2} \log \frac{4S_a}{3S_0}$$

	Source culture	Temperature	Log* bacterial Nos. (log B_0)	Rate of accretion (M)	Initial size (S_0)	Average adult size (S_a)	L† obs.	L‡ obs.	L calc. equation (4)
		°C.	*per ml.*	*doublings / hrs.*	$\times 10^{-7}$ *mm.³ / cell hr.*	$\times 10^{-7}$ *mm.³ / cell hr.*	*hrs.*	*hrs.*	*hrs.*
1	*Bact. dysenteriae*§	37.0	3.99	2.5‖	0.85	13.2‖	1.77	1.78	1.76
2	" " ¶	"	3.74	"	2.10	"	1.20	1.37	1.23
3	*Bact. coli***	"	5.92	3.1‖	15.1	16.1‖	0.19	0.19	0.16
4	" " §	"	3.72	"	0.85‖	"	1.50‖	1.51‖	1.51
5	" " ††	"	3.62	"	2.4‖	"	0.99‖	1.02‖	1.02
6	" "	32.5	4.15	2.4	"	15.8	1.32	1.32	1.31
7	" "	28.0	4.54	1.4	"	14.6	2.18	2.15	2.16

* Average of counts made prior to first sustained 10 per cent increase.

† Time required for 50 per cent increase in numbers

‡ Average of $L = t - nG$ for three or four successive generations; t = median of cell divisions, n = number of generations, $G = 1/M$.

§ Day old aerated broth cultures.

‖ Indicates average values, otherwise single experiments.

¶ 18 hour agar slopes.

** 3 hour broth culture. Equation (4) does not strictly apply to this case. Equation (3) should be used. See definition of "L" above.

†† Day-old stationary broth culture.

S_0 and S_a are expressed in terms of oxygen consumed at zero time in broth. See Methods.

These equations, therefore, describe the significant aspects of the growth and multiplication of any organism growing at constant rate and dividing by binary fission. If growth rate and net change in size are known, the latent period and the course of numerical increase can be predicted. For testing the assumptions outlined above, we have chosen to use the equations (3) and (4), since these are contingent on all the assumptions made,

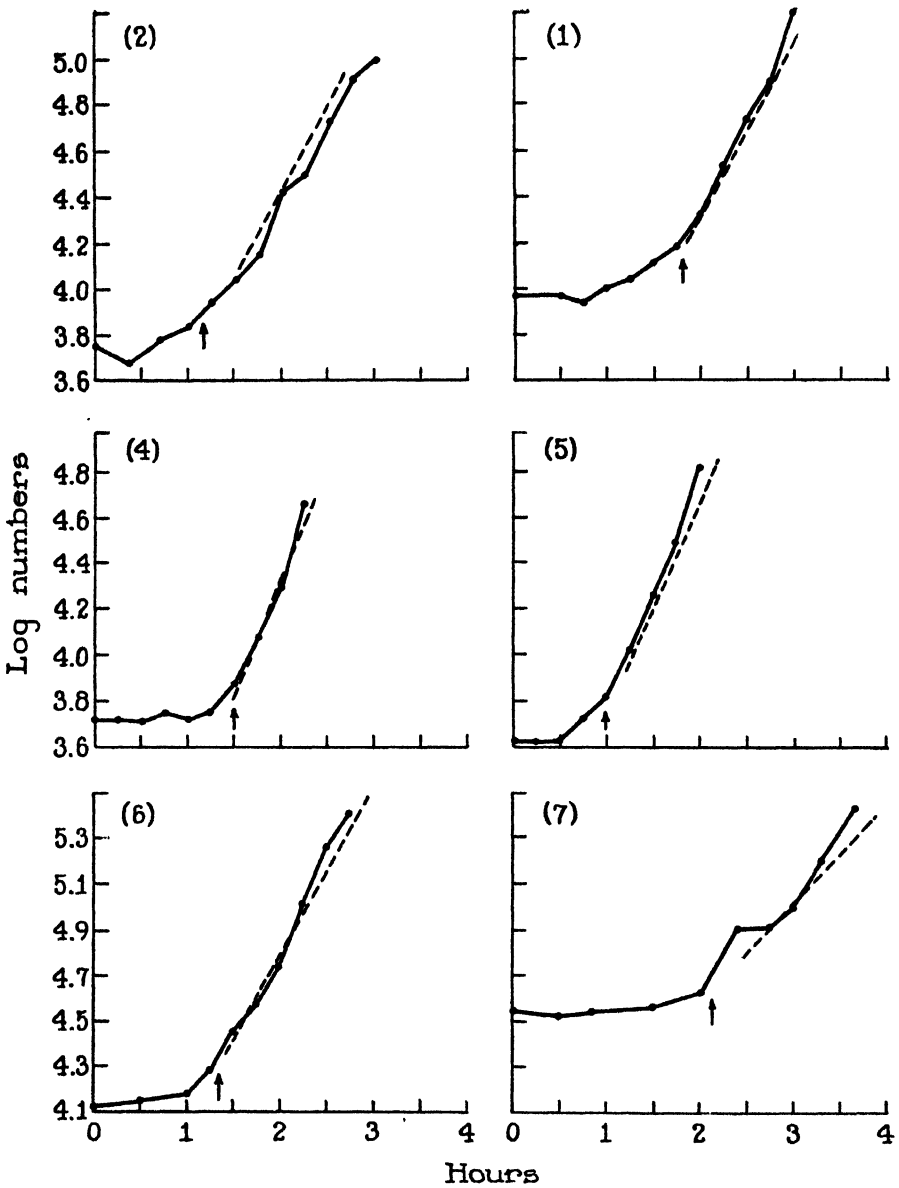

FIG. 1. Calculated and observed bacterial numbers. Equation (3): $\text{Log } B = \text{Log } B_0 + M\,t \log 2 - \log S_a/S_0$.

Lines connecting points are the experimental curves. The broken lines show the course of the calculated numbers. Arrows beneath the curves indicate the end of the observed latent period.

Values substituted in equation (3), and the conditions of the various experiments, are given in Table I.

and since measurements of B and L employ no new methods. All experiments have been carried out with *Bacterium coli*, excepting a few with *Bacterium dysenteriae* (Flexner).

The first problem we have considered is whether the growth of bacteria from young and old cultures, which are known to differ remarkably in size and latent period, is predictable from our hypotheses. That their growth-rates are substantially the same, can be regarded as established by the experiments recently published (2). The data given at that time fit equation (3) by definition, since the same measurements were used to compute size and growth rate. Nothing is to be accomplished, therefore, by re-examination of those data, which leave in question only the validity of the methods used.

This question is best answered by extending the observations to a considerable portion of the population curve, and assigning fixed average values to the postulated constants. New observations have accordingly been made, using conditions under which the rate of accretion and the initial size varied independently over a considerable range. Briefly, it was found that the latent period could be accurately and reproducibly measured, and that equation (4) satisfactorily predicted the times observed (Table 1). In the same experiments, the course of the numbers curve was accurately predicted by equation (3) in most instances (Fig. 1).

Whether the agreement obtained should be accepted as complete vindication of our assumptions remains, of course, a matter of opinion. Certainly their approximate truth has been established, as well as may be by measurements that are approximately correct.

An additional confirmation of hypothesis is given by the observation that population curves obtained with cells from aerated cultures (experiments 1 and 4, Fig. 1) show an abrupt transition from the latent period to the phase of logarithmic increase, as compared with cells from stationary broth or agar cultures (experiments 2 and 5). This peculiarity is less clear in curve 1, but has been observed in several additional experiments not shown. It is correlated with the remarkable uniformity of size revealed by microscopic examination, and perhaps by fractional sedimentation (3), of the aerated cultures, and should probably be referred to the homogeneous environment in which these cultures develop. The smaller absolute size of cells in aerated cultures is almost certainly the result of the virtually complete oxidative removal of food materials under these conditions.

Another observation bearing on the fundamental question of regulation

of size is that decreasing the growth rate by lowering the temperature diminishes the maximal size reached by *Bacterium coli*. Similarly, *Bacterium dysenteriae* apparently undergoes a smaller net change in size under the same conditions than the more rapidly growing organism. These suggestions are based on limited observations. In general it must be concluded that the mechanisms underlying the observed interdependence of growth rate, characteristic size, and environmental conditions (2), are unknown.

The values given in Table 1 include all measurements of L not previously reported. Experiment 4 represents the mean of three trials (L = 1.45, 1.53, and 1.53); experiment 5 of two (L = 0.95 and 1.02); the remainder are single observations. The data of Fig. 1 are also unselected, except that curves shown in the case of the duplicated experiments are those in which counting was continued longest.

Methods

The methods used were essentially those already reported (2). Population curves were obtained by agar-plate dilution counts, usually made at 15 minute intervals for accurate measurement of L. Dilutions were made in saline, with vigorous shaking, sufficient plates being poured to give 250 or more countable colonies (50 to 300 colonies per plate). Experimental variation has been reduced to an average of less than 10 per cent. Further improvement is apparently not feasible. Counts were conveniently made from cultures seeded with about 10^4 organisms per ml. The latent period obtained is little or not at all affected by the size of seeding within the stipulations noted (page 11). All cultures were incubated in a temperature-controlled water bath. A recording plate-counter (8) was indispensable.

Average values for the postulated constants, M and S_a, were used in all calculations. In the two experiments at the lower temperatures, these are averages of values observed at different times throughout the course of individual nephelometric experiments; in all others, they are averages of values obtained in numerous earlier experiments, for the most part manometric.

In current practice, minor technical improvements have been introduced leading to increased accuracy, particularly with the manometric method. For routine work, cells grown in day-old aerated broth cultures are being used to advantage, because these cultures are incapable of further growth, the oxidizable material available to the cells having been exhausted.

Aliquots of these cultures, for example, will give identical values for initial rate of O_2 consumption whether the period of equilibration is 20 minutes or an hour or more, prior to addition of fresh broth. Under the same conditions, the effect of oxygenation on stationary cultures is to initiate further growth, with or without added broth. Size is, therefore, measured nephelometrically, or after removing the cells from the culture medium.

The error resulting from inhibition of growth by removal of carbon dioxide during growth rate measurements (2) is largely avoided by placing the aliquot of the growing culture in the vessel for equilibration before adding the KOH. The vessel is then briefly removed from the bath for this purpose, after which readings can be made in 5 or 10 minutes.

The nephelometric method does not appear to be applicable to estimation of bacteria in day-old aerated cultures, giving values too high in relation to the viable count. The nephelometric index of size for these cultures is about 1.0, while the manometric size (0.85×10^{-7} mm.^{3}/cell hour) represents an index of 0.35 ($1.0 = 2.4 \times 10^{-7}$ mm.^{3}/cell hour). Since the latter figure agrees with microscopic observation,[4] we have given credence to the manometric value. Whether the excess turbidity is due to non-bacterial sediment, or dead bacterial cells, we have not determined. In either case, it could be expected to invalidate nitrogen determinations as well as optical measurements.

Improved measurements of growth rate with our strain of *Bacterium coli* at 37° C. have consistently given values between 3.0 and 3.3 doublings per hour, with an average of 3.1 (formerly (2) 2.8). This increase cannot be attributed to changes in the organism itself. Values for O_2 consumption given in this paper are based on new calibrations of the apparatus. Values previously reported are 16 per cent lower. This change is, of course, without effect on the values M and S_a/S_0.

[4] Mudge and Smith (9) report that *Bacterium coli* in aerated cultures is approximately one-third the length, at 24 hours, of the bacilli in stationary cultures. This is in accord with our own observation that the former are cocci, the latter bacilli about three times as long as broad, whose breadth equals the diameter of the cocci. Computing the volume of the bacillus as that of a cylinder 1×0.5 microns plus two hemispheres radius = 0.25 micron, the ratio of the volume of the bacillus to the coccus is 5.0. The corresponding ratio of manometric sizes is 2.4 to 0.85, or 2.8; of nephelometric sizes only 1.0. It may be recalled here that sizes reported by us are ultimately referred to nitrogen per cell, and presumably to volume; a bacillus about 1.5×0.5 microns = nephelometric index 1 = 2.4×10^{-4} mm.^{3}/cell hour = 3.5×10^{-11} mg. nitrogen per cell, it being necessary to establish the validity of each method for each new condition studied.

SUMMARY

Simple assumptions have led to equations by which the latent period in multiplication and the bacterial numbers expected at any time during the phase of rapid growth may be predicted.

Experimental data obtained under rather diverse conditions have given satisfactory agreement with calculated values. Since the mathematical expressions contain no arbitrary constants, more than accidental significance must be attached to this agreement.

The hypotheses set forth appear completely to describe the early development of *Bacterium coli* and *Bacterium dysenteriae* in broth, without postulating differences other than size among individual cells, or cells obtained under different conditions.

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REGENERATION OF VISUAL PURPLE IN SOLUTION*

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I

INTRODUCTION

Regeneration of visual purple in solution was originally observed by Kühne (Ewald and Kühne, 1878). It was not until recently that it was again reported by Hecht, Chase, Shlaer, and Haig (1936). They found by measuring density changes at 500 mμ with Shlaer's photoelectric spectrophotometer (Shlaer, 1938) that regeneration occurred only after about 85 per cent of the visual purple originally present had been bleached and that it was maximal in solutions of pH about 7.7. Hosoya (1938) has reported that under certain conditions visual purple can be made to regenerate in solution after only a relatively small percentage of it has been bleached.

It is known that at neutral and acid pH's the photic decomposition of visual purple results in the appearance of other colored compounds (Weigert and Nakashima, 1930; Chase, 1936; Lythgoe, 1937), one or more of which become colorless at a rate dependent upon the temperature (Hosoya, 1933; Wald, 1937; Lythgoe, 1937). Since visual purple regeneration is measured optically, the presence of such additional color changes would complicate and perhaps mask the changes of visual purple itself at these pH's. Because this effect becomes greater in more acid solutions, it was apparent that pH 7.7 might not be the true maximum for regeneration.

We have extended the investigations already reported by examining in detail the effect of pH on regeneration, taking into consideration the formation and disappearance of colored decomposition products, and making the measurements in such a way as to minimize the complicating factors that are present in neutral and slightly acid solutions. We have also investigated the effect of wave length of the bleaching light, as well as that of temperature during extraction.

We have secured data which give information about the course of the regeneration reaction in solution by measuring the absorption spectrum of solutions during regeneration, and have made some investigations into the kinetics of the chemical reaction which controls the process.

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II

Materials and Methods

The visual purple was obtained from frogs (*Rana pipiens*) freshly received from Alburg, Vermont. In general, the methods of extraction were as already described (Chase and Haig, 1938). The retinas were usually hardened by immersing in 4 per cent alum solution for 2 hours before being put in the extractive, although this step was omitted in some cases. Unless otherwise specified, the extractions were made at room temperature, 20 to 24°C. All centrifuging was done at 6°C., in order to minimize decomposition of the visual purple during the periods of 1½ to 3 hours in the centrifuge, whose temperature often reaches 30° or slightly higher if no effort is made to prevent its rise.

About 12 to 16 retinas were used for each milliliter of the extractive. Either 4 per cent purified bile salts solution or 2 per cent digitalin solution (digitalin cryst., Eimer and Amend), was used, usually the latter because of its greater freedom from bacterial growth. The resulting solution, after addition of 20 per cent (by volume) of buffer solution, had an optical density at 500 mμ of about 1.8 in a depth of 20 mm. The solutions were always used as soon as possible after their preparation in order to prevent artefacts caused by changes which take place upon standing, even at low temperatures. In most cases the solutions were not more than 48 hours old; often not older than 24 hours.

A special absorption cell was used which held only 2½ to 3 ml. of solution and had an optical depth of 20 mm. The cell was rectangular and its interior cross-sectional area was only slightly greater than the cross-sectional area of the monochromatic measuring beam of the spectrophotometer.

In all our measurements we used Shlaer's photoelectric spectrophotometer (Shlaer, 1938). The data are given as photometric densities; these are equal to log (I_0/I_t) where I_0 is the intensity of the incident beam and I_t that of the beam transmitted by the solution. The photometric density is directly proportional to the concentration of absorbing substance in the solution.

As has been already shown (Chase and Haig, 1938) it is essential when studying the effect of any variable on the behavior of visual purple in solution, to use identical samples of one extraction of visual purple. This practice was consequently followed in the experiments to be reported. Each extraction was divided into several samples and these samples were then treated and measured at the same time, or as nearly the same time as possible—usually within 1 day—so that the solution itself should not undergo changes between measurements on the different samples.

III

Effect of Duration and Wavelength of Exposure

Photic decomposition of visual purple at neutral and acid pH's results in the formation of a yellow material which itself fades to a more or less colorless condition (Hosoya, 1933; Wald, 1937; Lythgoe, 1937). Therefore, when studying visual purple regeneration it is necessary to illuminate for a sufficiently long time to allow the yellow material initially formed to bleach

before the regeneration measurements are begun. Otherwise the density change measured will be the algebraic sum of a decrease in density caused by the fading of yellow color and an increase in density caused by regeneration of visual purple, with the result that the visual purple regeneration will appear less than it actually is and the shape of the regeneration curve will not be significant as far as visual purple is concerned.

Fig. 1 illustrates this effect under extreme conditions which favor its appearance. Two samples of visual purple solution, one of pH 9.9 and the other of pH 5.2, were illuminated for 5 seconds with a photoflood lamp at 3 inches distance and their density at 480 mμ was then measured for 2 hours in the dark. In both samples the density decreases during the 2 hour period but the total density fall is about 10 times as great and its rate much less in the acid sample than in the alkaline sample. The fading of this decomposition product is of course less of a factor in regeneration measurements when the exposure time is longer. All of the exposures used in the experiments to be reported were therefore of long duration (3/4 to 1-1/2 hours) as contrasted with the 10 minute exposure time used earlier (Hecht, Chase, Shlaer, and Haig, 1936).

It has already been shown (Chase, 1937) that the spectral content of the bleaching source greatly influences the subsequent visual purple regeneration. If the illuminating source contains blue and violet much greater regeneration follows the bleaching than when these wavelengths are lacking. This was demonstrated by dividing a visual purple solution into two samples and bleaching one with light containing only the longer visible wavelengths while the other was bleached with light from the short-wave part of the visible spectrum. Although the two sources were adjusted in intensity to be equally effective for bleaching the visual purple in the solutions, much more regeneration occurred in the sample bleached by the source containing the blue and violet than in the other sample. Moreover, in the sample bleached by the blue and violet source there was a much greater density decrease at the shorter wavelengths during the illumination than there was during the illumination of the other sample with light containing red, orange, and yellow. This latter sample, upon being re-illuminated, but this time with blue and violet light, showed a considerable decrease in density at the shorter wavelengths and, after the light had been turned off, regenerated as much as the other sample had done. These results indicated that visual purple solutions contain a photosensitive substance whose breakdown by light is necessary before visual purple regeneration can take place. An experiment of the sort described is illustrated in Fig. 2, where density is measured at two wavelengths on two samples.

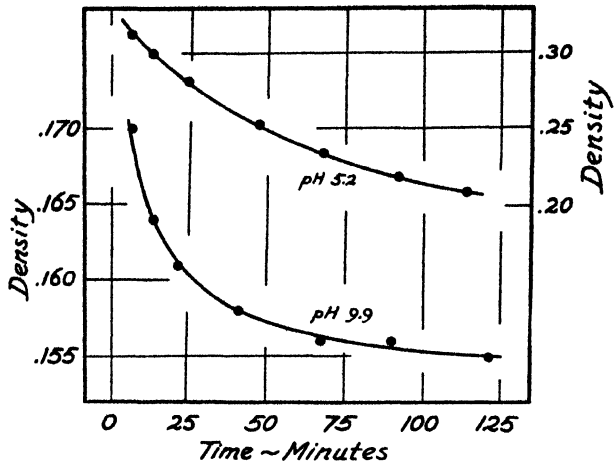

FIG. 1. The fading of two visual purple samples measured at 480 mμ after a 5 second exposure to a photoflood lamp. The decrease in density is less but much faster at the more alkaline pH. In regeneration studies, illumination must be sufficiently long to allow these processes to complete themselves before density measurements are begun.

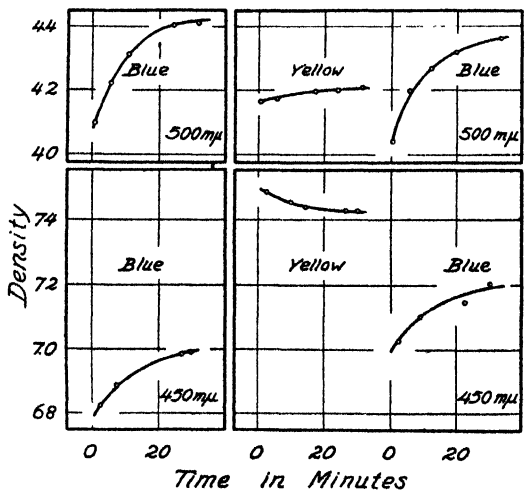

FIG. 2. Influence of the color of the bleaching source on visual purple regeneration. The sample on the left was bleached with predominantly blue and violet light; it shows distinct regeneration, both at 500 mμ and 450 mμ. The sample on the right was bleached with light which lacked blue and violet; it shows only slight regeneration at 500 mμ and fading at 450 mμ. A second bleaching of the latter sample with blue and violet light causes a large density decrease, particularly at 450 mμ, and a subsequent regeneration as great as that given by the sample shown on the left.

It was possible to obtain an approximate measurement of the absorption spectrum of this photosensitive material by considering the visual purple regeneration which followed bleaching with different wavelengths as a criterion of the absorption of these different wavelengths by the unknown photosensitive substance. Identical samples of a visual purple solution were bleached with white light filtered by four different Wratten monochromatic filters, Nos. 73, 74, 75, and 76. By means of neutral filters the intensity of these colored sources was adjusted until they were equally effective in bleaching a sample of the visual purple solution identical with those used in the experiments. When the effective intensity of the colored sources had been equalized in this way, each one was used to illuminate a single sample of the solution for 3/4 of an hour and the amount of regenera-

TABLE I

Influence of Wavelength of Bleaching Light on Subsequent Regeneration

The sources were adjusted to be equally effective in bleaching the visual purple. Exposure time was 45 minutes for the wavelengths isolated by the Wratten filters. The regeneration values given are per cent of density change at 500 mμ. The pH was 7.6.

Filter No. and central λ of bleaching light		Regeneration	
		Solution A	Solution B
	mμ	*per cent*	*per cent*
(No. 76)	440	2.9	2.9
(No. 75)	490	3.3	1.6
(No. 74)	525	1.4	1.7
(No. 73)	575	0.3	0.3

tion that occurred was measured at 500 mμ in each case. The results of two such experiments are given in Table I. The values are per cent regeneration, calculated as density regenerated at 500 mμ divided by the density at that wavelength of the visual purple originally present. The values given for the bleaching wavelengths represent the centers of the fairly narrow spectral bands transmitted by the so called monochromatic filters.

Although the per cent of regeneration that occurred in these experiments was small (probably because the bleaching intensities were limited by the low transmission of the filters), the differences in per cent regeneration following the various illuminations are quite significant, and leave no doubt that the substance whose photic decomposition is responsible for visual purple regeneration has an increasing absorption toward the shorter wavelengths and is consequently yellow in color. This yellow substance may

be a decomposition product of visual purple itself. It is also possible that a flavin—known to be present in the eye—is involved, but we have so far not been able to demonstrate this. Granit and Wrede (1937) have postulated, as a result of analysis of electroretinograms, a pigment or receptor in the frog's eye which reacts specifically to blue and violet light, so it is not impossible that this photosensitive yellow pigment may have a rôle in vision apart from aiding visual purple regeneration.

IV

Effect of pH on Regeneration

In measuring regeneration of visual purple solutions at different pH's we used three different visual purple extractions, each one furnishing three to six samples. These samples were adjusted to the desired pH values with Clark and Lubs buffers (Clark, 1928), made up to be 0.4 M. They were added to the visual purple solutions in the proportion of one part of buffer to four parts of solution, by volume. The pH of the resulting solutions was checked with a glass electrode. A 500 watt projection lamp at 10 inches served as the bleaching source. This insured adequate energy, and a 45 minute exposure allowed most of the intermediate yellow color to disappear before the regeneration measurements were begun. A heat-absorbing glass filter was interposed between the lamp and the solution.

The density of each solution was measured in the dark for three-quarters of an hour immediately following the illumination. Measurements were made at three wavelengths, 440, 500, and 560 mμ. Because the transient yellow materials in the solution absorb only slightly, if at all, at 560 mμ, the density changes at this wavelength were taken as representative of visual purple regeneration. After measuring regeneration for 45 minutes, the solutions were illuminated for 15 minutes more and again measured to make sure that the density change which had been observed was really caused by the building up in the dark of photosensitive substances and not simply by increases in turbidity or other artefacts.

The density values at 560 mμ following the illumination were plotted against time in the dark and smooth curves drawn. From these curves were taken the density values for 5 minutes and 30 minutes in the dark so as to avoid errors of extrapolation, and the difference between these two values was taken as a measure of the regeneration that had occurred at any given pH.

Because these samples were from three extractions of different visual purple concentration, they did not show the same amounts of regeneration.

Each set of samples was therefore used to construct a single curve showing the relation between pH of the solution and visual purple regenerated.

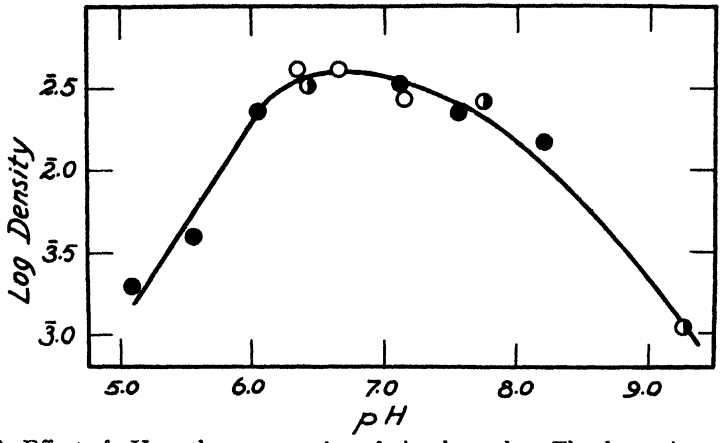

FIG. 3. Effect of pH on the regeneration of visual purple. The data, given in Table II, are from three different solutions, and represent measurements at 560 mμ.

TABLE II

Effect of pH upon Amount of Regeneration

Regeneration measured by the change in density at 560 mμ, after bleaching for 45 minutes with a 500 watt projection lamp at 10 inches from the solution. Data of Fig. 3.

Solution	pH	Density values from smoothed curves			Log of difference	Adjusted log values
		5 min.	30 min.	Difference		
A	5.09	0.136	0.138	0.002	3̄.301	3̄.301
	5.55	0.153	0.157	0.004	3̄.602	3̄.602
	6.04	0.148	0.171	0.023	2̄.362	2̄.362
	7.12	0.158	0.192	0.034	2̄.532	2̄.532
	7.69	0.101	0.124	0.023	2̄.362	2̄.362
	8.19	0.122	0.137	0.015	2̄.176	2̄.176
B	6.42	0.093	0.108	0.015	2̄.176	2̄.520
	7.74	0.070	0.082	0.012	2̄.079	2̄.420
	9.25	0.0860	0.0865	0.0005	4̄.699	3̄.050
C	6.35	0.174	0.186	0.012	2̄.079	2̄.620
	6.67	0.156	0.168	0.012	2̄.079	2̄.620
	7.16	0.136	0.144	0.008	3̄.903	2̄.440

Since the three visual purple extractions presumably differed only in visual purple concentration, it was possible to plot the logarithms of the density changes observed and obtain three curves, which were of essentially the

same shape, but lay at different levels on the log density axis. By shifting two of these curves vertically a single curve results about which all the points fall. This curve is plotted in Fig. 3. The experimental density values used to construct the curve are given in Table II, together with their logarithmic values and the adjusted logarithmic values which appear in the figure. Over the pH range from 5.0 to 9.6 a maximum for regeneration is shown at about pH 6.7, a value more acid than that earlier reported from this laboratory by Hecht, Chase, Shlaer, and Haig (1936). The reason for this difference is that in the present measurements allowance was made for the disappearance of transient yellow intermediate products.[1]

It is quite possible that the visual purple molecule or something else in the solution essential for regeneration may undergo dissociation with changes of hydrogen ion concentration and thus exert an effect upon regeneration. If this is so, we might expect that the curve relating regeneration to pH should resemble a titration curve. The curve of Fig. 3 does not, but it is possible that decomposition or the presence of colored products at pH's below 6.6 prevents the measurement of regeneration there which may theoretically occur. The abrupt drop in the curve on the acid side suggests some sort of decomposition. It is also possible that the curve of Fig. 3 may represent by its maximum an isoelectric point or some other property of a protein, since visual purple is now generally recognized to be protein in nature. The data are neither complete nor precise enough to warrant more than a mention of such possibilities. They do show that under practical conditions the maximum amount of measurable regeneration occurs at a pH of about 6.7.

V

Effect of Extraction Temperature

If some other substance besides visual purple is necessary for regeneration to occur after illumination and if this substance is extracted from the retina along with the visual purple, different temperatures during extraction might

[1] The influence of this factor is very great as can be shown if the visual purple regeneration is measured at regions in the spectrum where the unstable colored products of visual purple decomposition have a greater absorption than they have at 560 mμ. Thus, if we had taken the amount of regeneration measured at 500 mμ instead of at 560 mμ, the curve relating regeneration to pH would have been found to have its maximum at about pH 7.1 instead of pH 6.7 and there would have even been some decrease rather than increase in density at pH's below 5.6. When the regeneration as measured at 470 or 440 mμ is used, the effect of masking reactions is even more marked. Because it is as yet impossible to eliminate completely such factors, the pH maximum for regeneration may even be somewhat further toward the acid side.

be expected to change the ratio of visual purple to this other material, with a consequent difference in the amount of regeneration in the resulting solutions.

Retinas from 150 frogs were prepared for extraction in the usual way. These were then divided into three equal lots and extracted simultaneously at 7°, 20°, and 35°C., respectively. The resulting solutions were buffered at pH 6.7 and measured to determine their visual purple density. A sample of each was then illuminated for 45 minutes and measured at 500 mμ for 45 minutes immediately following the illumination. The density regenerated at 500 mμ for each sample during the 45 minute period was divided by the density at that wavelength of each sample before illumination. The sample from the 7° extraction showed 9.8 per cent regeneration, that from the 20° extraction showed 9.9 per cent regeneration, and the 35° sample regenerated 9.2 per cent. These values are sufficiently alike to show that temperature variation over the range between 7° and 35°C. during extraction is without effect upon regeneration of visual purple. Therefore, if another substance necessary for regeneration must be extracted from the retina, the solubility of this hypothetical substance in 2 per cent digitalin solution varies with temperature in the same way as that of visual purple, unless both go completely into solution at all the temperatures studied.

VI

Absorption Spectra during Regeneration

The absorption spectrum of a bleached visual purple solution was measured at different times during regeneration in order to determine whether any other reactions involving color changes occur.

It is impossible with this spectrophotometer to measure completely an absorption spectrum which is changing rapidly. We therefore adopted the following method. A visual purple solution buffered at pH 7.6 was illuminated for 1 hour with a 100 watt lamp and immediately after turning off the light the density of the solution was measured at 500 mμ, 470 mμ, 440 mμ, and 530 mμ. The total time required to measure the density at these four wavelengths was about 5 minutes. The density at 500 mμ was then measured again, followed by that at 470, 440, and 530 mμ, while the visual purple was regenerating, and this process was continued for 2 hours. The solution was then exposed for 15 minutes to the bleaching source and the density at the four wavelengths again determined to insure that any observed changes in the dark had been caused by regeneration of photosensitive substances. Curves were constructed showing density changes

in the dark for each of the wavelengths measured. The data used in the construction of these curves are given in Table III.

From the curves it was easy to calculate the density increase at each of

TABLE III

Course of Regeneration of Visual Purple at pH 7.6 Measured in Different Parts of the Spectrum

440 mμ		470 mμ		500 mμ		530 mμ	
Time	Density	Time	Density	Time	Density	Time	Density
min.		*min.*		*min.*		*min.*	
(0.0)	(0.856)	(0.0)	(0.602)	(0.0)	(0.435)	(0.0)	(0.332)
1.0	0.860	2.0	0.616	3.5	0.465	5.0	0.356
7.0	0.884	8.5	0.651	10.0	0.504	12.0	0.380
14.0	0.900	15.0	0.674	17.0	0.527	18.0	0.394
26.0	0.918	28.0	0.700	30.5	0.558	32.5	0.414
36.5	0.928	38.0	0.715	40.0	0.571	42.0	0.424
48.0	0.935	50.0	0.726	51.5	0.583	53.5	0.432
61.5	0.938	64.0	0.736	65.0	0.593	68.0	0.438
92.3	0.948	78.0	0.744	80.5	0.601	82.5	0.444
97.0	0.950	99.0	0.750	100.5	0.610	103.5	0.449
119.0	0.952	121.0	0.756	122.5	0.616	125.0	0.453
(∞)	(0.956)	(∞)	(0.760)	(∞)	(0.622)	(∞)	(0.456)

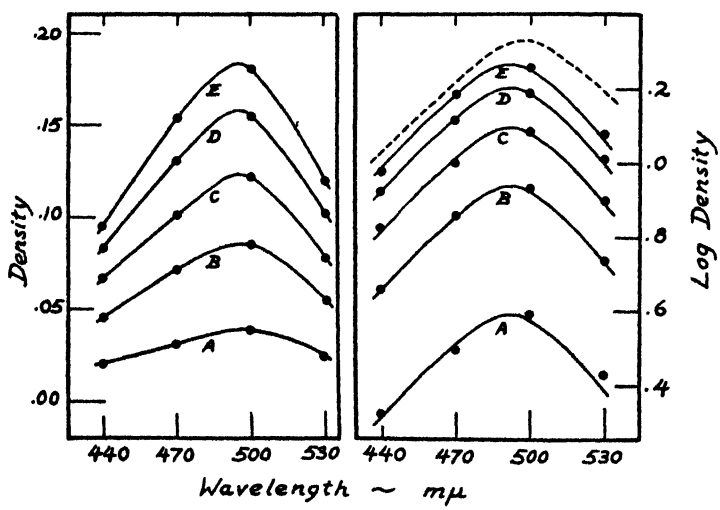

FIG. 4. The spectrum of the regenerating color at different times after bleaching; A, 5; B, 15; C, 30; D, 60; and E, 120 minutes. The log density curves on the right have the same shape and therefore show only concentration differences. The absorption spectrum of a partially purified visual purple solution before illumination is shown by the dash-line curve in terms of log density for comparison. Data are in Table III.

the five wavelengths during different times in the dark, obtaining the density for zero time in the dark by extrapolation of the curves. These values for zero time are also shown in the table, as are the final density values reached, also obtained by extrapolation.

The absorption spectrum of the regenerated material was computed for 5, 15, 30, 60, and 120 minutes in the dark and these five spectra are plotted in the left half of Fig. 4. If visual purple is the only substance that appears during the dark period the absorption spectrum constructed from the regeneration curves should be identical with the absorption spectrum of visual purple. If another colored substance (or substances) is formed at the same time but at a different rate the absorption spectra measured at different times during regeneration should not represent visual purple alone, nor should they be multiples of each other. This should also be the case if visual purple regeneration first involves the formation in the dark of some other colored substance, which then takes part in building visual purple.

It is clear that the absorption spectra of Fig. 4 are not very different from that of visual purple although there is slightly more absorption in the blue and violet than should be expected. A simple way of showing similarity or dissimilarity of these five absorption spectra among themselves is to plot them in their logarithmic form. Such curves, being independent of concentration differences, can be shifted on the log density axis. They can be superimposed only if they differ from one another in concentration alone, and not if two or more reactions involving color changes are occurring at different rates. Such logarithmic plotting is shown in the right half of Fig. 4. The curves drawn through the five sets of points are identical and it is therefore apparent that only one principal colored substance is being built up in the dark.

The logarithmic form of the absorption spectrum of a partially purified unbleached visual purple solution is also shown in Fig. 4 by the dash-line curve to emphasize the small departure of the absorption spectrum of the regenerated substance from that of visual purple. It is possible that this small extra absorption represents the simultaneous regeneration in the dark of the yellow photosensitive material described in section III. However, several yellow pigments are now known to appear in visual purple solutions so it is not at present profitable to try to identify this extra absorption at the shorter wavelengths, especially as its occurrence is rather variable.

In another experiment conducted at pH 6.7 and involving density measurements at 440, 470, 500, 530, and 560 mμ, measuring at 500 mμ and two other wavelengths on each of two identical samples, the same results were obtained except that the extra absorption at the shorter wavelengths

was greater. Still other experiments agreed in this respect with the one illustrated. It is possible that the greater absorption at shorter wavelengths in the more acid solutions may be caused by a natural acid-base indicator in the regenerating solution, as Chase has shown occurs during bleaching of visual purple (Chase, 1936), but the data are not conclusive on this point. It is also possible that visual purple regenerated in solution is not the same as the extracted visual purple before illumination.

Visual purple regeneration in solution then does not occur through consecutive reactions involving color changes, unless such changes take place during the initial bleaching of the visual purple, or during the first few moments in the dark, too rapidly to be measured.

The occurrence of this extra absorption at the shorter wavelengths in the case of the regenerating visual purple, offers an obstacle to the calculation of regenerated visual purple in terms of per cent of that present in the unilluminated solution. If the density of the regenerated material at 450 mμ, for example, is compared with the density at that wavelength of the visual purple before illumination (using either the classical absorption spectrum or the absorption spectrum of the unbleached solution), the apparent percentage regeneration may be as much as twice as great as it is if these calculations are made using the density measured at 500 mμ.

Having recognized this fact, it is nevertheless possible to use percentages of regeneration as a means of comparing the regeneration capacity of various extractions, provided that calculations are always made in terms of a single wavelength, preferably 500 mμ. Measured at this wavelength and in solutions of the optimum pH, regeneration during the current work was found to vary in different extractions from 6 per cent to 18 per cent, with 12 per cent the value most often encountered. The greatest amount of regeneration which we obtained involved a density increase at 500 mμ of about 0.18 in a solution which had a visual purple density of 1.0 before illumination. The optical depth of the absorption cell in this case was 10 mm.

VII

Successive Regenerations

Kühne reported (Ewald and Kühne, 1878) that both in the isolated retina and in solution visual purple could be made to undergo several successive regenerations, each time to a less extent, until finally none occurred. In repeating his observations we were interested to see whether the gradual failure to regenerate after successive bleachings was caused by the disappearance of a reactant or by the destruction of a catalyst of some sort.

If a catalyst is failing, the second and third regenerations should be at a slower rate than the first, but should ultimately produce the same concentration of visual purple. On the other hand, if a reactant is being used up, the amount of visual purple regenerated should be less in the second regeneration than in the first, and still less in the third, but the curves for all three should be of the same form. We illuminated a visual purple solution (buffered at pH 7.7) for 3/4 hour and then measured the density change at

TABLE IV

Successive Regenerations of Visual Purple

Density measurements at 500 mμ. The first bleaching was for 45 minutes; the second and third were for 15 minutes each. pH 7.7. Density values for zero and infinite regeneration time were obtained by extrapolation of the smoothed curves. These data are plotted in Fig. 5.

First regeneration		Second regeneration		Third regeneration	
Time	Density	Time	Density	Time	Density
min.		*min.*		*min.*	
(0.0)	(0.0775)	(0.0)	(0.0775)	(0.0)	(0.0775)
0.7	0.0812	1	0.0804	5	0.0860
4	0.0872	5	0.0872	10	0.0900
7	0.0968	11	0.0948	17	0.0956
12	0.1056	18	0.1024	28	0.1008
19	0.1152	25	0.1076	42	0.1052
26	0.1244	31	0.1120	51	0.1076
33	0.1304	39	0.1160	64	0.1104
40	0.1352	51	0.1204	79	0.1124
49	0.1404	64	0.1252	100	0.1152
62	0.1456	72	0.1272	113	0.1164
70	0.1496	82	0.1284	119	0.1172
80	0.1536	87	0.1300		
89	0.1556	96	0.1320		
100	0.1580	108	0.1332		
110	0.1600	115	0.1344		
117	0.1612	120	0.1352		
(∞)	(0.1675)				

500 mμ for 2 hours in the dark. The solution was then re-illuminated for 15 minutes and measured in the dark for another 2 hours. After this it was illuminated a third time for 15 minutes and again measured for 2 hours in the dark. Fig. 5 shows the results of this experiment, the data being given in Table IV. The two curves through the lower sets of points are calculated multiples of the smooth curve drawn through the upper set of points. The fact that the experimental points fall upon these calculated curves so well

indicates that in successive regenerations a reactant is used up and an enzyme or catalyst apparently is not the controlling factor. It is possible that the reactant in question is normally supplied from the pigment layer as Kühne believed (Ewald and Kühne, 1878). In such a case there would of course be only a limited quantity of it in the solution, which should cause exactly the behavior observed in this experiment.

The behavior of visual purple which has been extracted from the retinas by 4 per cent sodium desoxycholate solution may be significant in this connection. Visual purple solutions obtained by use of this extractant do not regenerate, even at pH's that are optimal for bile salts and digitalin extractions. The simplest explanation is that the sodium desoxycholate combines with the visual purple molecule or with its decomposition products in such a way that rebuilding of the visual purple after illumination does not take place. Alternatively, the desoxycholate may combine with or fail to extract from the retina some other substance which normally enters into the reactions through which visual purple regeneration is accomplished.

VIII

Reaction Kinetics

The reaction kinetics of the bleaching of visual purple by light have been shown to be first order (Hecht, 1921; Chase, 1936). Regeneration of visual purple, on the other hand, might be expected to be the result of a bimolecular reaction, especially in view of the dependence of dark adaptation on vitamin A (Fridericia and Holm, 1925; Tansley, 1931; Hecht and Mandelbaum, 1938; Wald, Jeghers, and Arminio, 1938). However, if one of the reactants were present in any great excess, a first order equation should describe the data.

The precautions necessary in order to favor the maximum amount of visual purple regeneration, uncomplicated by the simultaneous appearance or disappearance of other colored substances, have been mentioned. The absorption of these substances is greater at shorter than at longer wavelengths so that density measurements made at 560 mμ should be expected to be more indicative of changes in visual purple concentration, uncomplicated by concentration changes of yellow substances, than would measurements made at 500 mμ. On the other hand, measurements made at 560 mμ would be subject to larger experimental errors than measurements made at 500 mμ because the absorption of visual purple is much less at 560 mμ than at 500 mμ. We therefore measured the density change at several wavelengths simultaneously following an exposure of 1 hour, which allowed

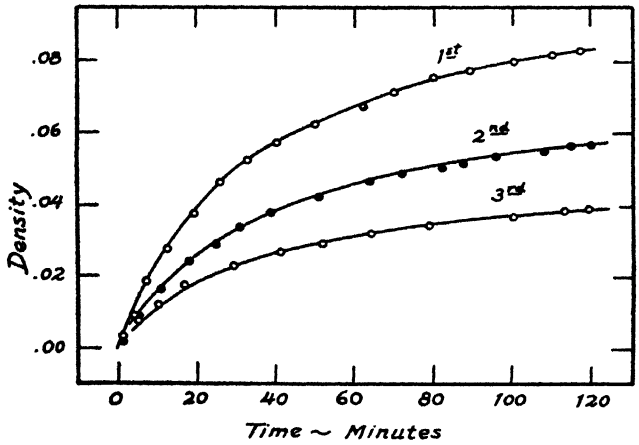

FIG. 5. Three successive regenerations in the same solution, measured at 500 mμ. The colored material bleaches to the same density each time and the three curves are multiples of one another. The data are given in Table IV.

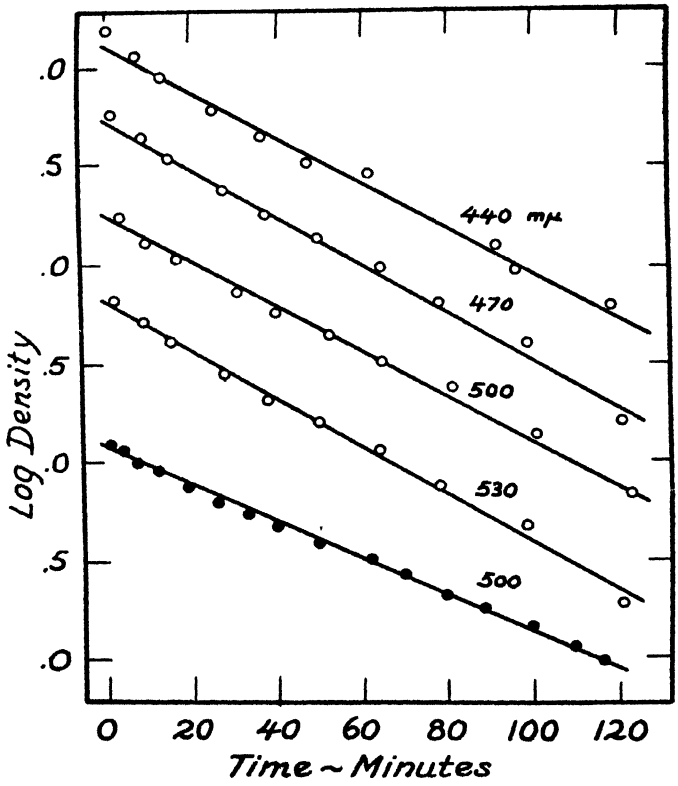

FIG. 6. Description of visual purple regeneration by the first order equation. The four upper sets of data are from Table III; the lowest set (solid black circles) are from the first regeneration given in Table IV.

most of the fading reaction to complete itself before the light was turned off and the measurement of regeneration was begun.

The data of Table III are typical of a number of measurements of visual purple regeneration. If visual purple is formed from its precursors in the illuminated solution, then if the reaction is monomolecular, or if it is bimolecular with one reactant present in excess, the first order equation should describe the regeneration process.

In the equation, $Kt = \log \frac{a}{(a - x)}$, x represents the concentration of visual purple formed during the time in the dark, t, and a represents the concentration of reactant present at zero time. This quantity, a, can then be assumed to be equal numerically to the difference between the density of the solution at the end of the reaction and that at the beginning. $(a - x)$ can be calculated for any time, t, by subtracting the density regenerated at that time from the constant value of a.

If the logarithm of $(a - x)$ is plotted against t a straight line should describe the data if the reaction is first order. Fig. 6 shows the data from Table III and from the first double column of Table IV plotted in this way. It is apparent that the first order equation describes the regeneration of visual purple in solution fairly well. The small deviations which appear must represent complicating factors that are not understood at present since they look regular rather than random. For an approximate description of visual purple regeneration, however, the first order equation is quite adequate.[2]

IX

Retinal Metabolism and Regeneration

Warburg and Negelein (1929), Kubowitz (1929), and Nakashima (1929) studied the metabolism of the retinas of white rats, frogs, and fish, and failed to report any effect of light. More recently Jongbloed and Noyons (1936) have presented data which indicate that the oxygen consumption and CO_2 production of frog half-eyes are increased about 25 per cent in the dark fol-

[2] In a few measurements of visual purple regeneration the data could not be described by the first order equation nor by that of a second order reaction. Inspection of such curves always showed an abnormally high rate of density increase during the first 15 minutes of the dark period with the change practically over by the end of one-half hour, whereas it usually continues for 2 hours or longer. It seems unprofitable to attempt an explanation of these infrequent cases until more is known of the other color changes that may occur during visual purple regeneration.

lowing an exposure to light. They concluded that the excess metabolism is probably concerned with the resynthesis of visual purple.

In 1934, one of us (E. L. S.) began a series of respiration experiments with frog retinas (*Rana pipiens*). Measurements were made with the Warburg apparatus, using from 6 to 8 retinas or half-eyes in vessels of about 8 ml. capacity at temperatures of 25° and 30°C. No detectable differences in oxygen consumption were found between light and dark retinas under a variety of conditions. Measurements were made with isolated retinas in Ringer's solution and also with half-eyes in vitreous humor. Changes in pH from 5.0 to 9.0 had no effect on the oxygen consumption in Ringer. These observations were all made with summer frogs. Since Jongbloed and Noyons worked with winter frogs some of the measurements were repeated during the winter of 1937–38 with the same results as before.

The illumination system involved a 500 watt lamp with a condensing system, so that a high light intensity was available. Measurements were made with intensities of from 1000 to 100,000 meter candles. In some experiments, after a bleaching period of 15 minutes the eyes were removed from the manometer vessel and the visual purple was found to be completely bleached out. Other half-eyes were subsequently measured over a period of 2 hours in the dark. When these were removed they showed a definite pinkish color in the retina, indicating that regeneration of visual purple had taken place, although not completely as compared with freshly extirpated retinas from dark-adapted frogs.

If any increase in oxygen consumption occurs in the retinas of *Rana pipiens* concurrent with the regeneration of visual purple, it cannot be larger than 5 per cent.

SUMMARY

1. Measurements of visual purple regeneration in solution have been made by a procedure which minimized distortion of the results by other color changes so that density changes caused by the regenerating substance alone are obtained.

2. Bleaching a visual purple solution with blue and violet light causes a greater subsequent regeneration than does an equivalent bleaching with light which lacks blue and violet. This is due to a photosensitive substance which has a gradually increasing effective absorption toward the shorter wavelengths. It is uncertain whether this substance is a product of visual purple bleaching or is present in the solution before illumination.

3. The regeneration of visual purple measured at 560 mμ is maximal at about pH 6.7 and decreases markedly at more acid and more alkaline pH's.

4. The absorption spectrum of the regenerating material shows only a concentration change during the course of regeneration, but has a higher absorption at the shorter wavelengths than has visual purple before illumination.

5. Visual purple extractions made at various temperatures show no significant difference in per cent of regeneration.

6. The kinetics of regeneration is usually that of a first order process. Successive regenerations in the same solution have the same velocity constant but form smaller total amounts of regenerated substance.

7. *In vivo*, the frog retina shows no additional oxygen consumption while visual purple is regenerating.

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THE KINETICS OF PENETRATION

XX. Effect of pH and of Light on Absorption in Impaled Halicystis

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In the present paper experiments are described in which impaled *Halicystis* cells were exposed to sea waters of different pH under ordinary lighting conditions, and to sea water of normal pH under different lighting conditions.

These experiments were carried out at the Bermuda Biological Station in the winter of 1936–37 on *Halicystis Osterhoutii*, Blinks and Blinks.

EXPERIMENTAL

In the pH experiments cells, after a preliminary exposure to sea water of normal pH (8.2) for 5 days, were exposed to sea waters of which the pH's were 7.0, 8.2 (control), and 9.2. Each cell used was exposed successively to all three sea waters. During the exposure the rate of increase in volume was determined by measurement of the rise of liquid in a tube of capillary bore on which the cell was impaled. The method of doing this and the arrangement of the cells for this type of experiment has already been described.[1] In these experiments, as before, the bottles containing the cells were kept at 17°C. ± 1°C. by means of a flow of water from the Biological Station's circulating salt water system. The experiments were carried out in a dark basement room in order to avoid to some extent alterations in the pH of the surrounding sea water due to photosynthesis.

The pH of the sea water was adjusted to the neighborhood of 7.0 by adding small successive portions of 0.6 M HCl with vigorous aeration between additions to blow out the excess CO_2 formed. In the case of the pH 9.2 sea water, some 0.6 M Na_2CO_3 was added with subsequent aeration. The pH remains fairly constant at 7.0 if the sea water is kept in a pyrex bottle open to air. But at the higher pH it tends to fall slowly due probably to the loss of CO_3 ion in the form of magnesium and calcium carbonates.

In contact with cells the pH of the low pH sea water tends to rise to that of the control, and the pH of the high pH sea water to fall off to that of the control. It is possible to hold the pH constant by adjusting the free CO_2 concentration of the sea water but this step seems to us to be an undesirable one since free CO_2 passes rapidly through the protoplasm while the ions do not[2] and the pH of the sap is disturbed seri-

[1] Jacques, A. G., *J. Gen. Physiol.*, 1938–39, **22**, 147, 757.

[2] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 695.

ously by this procedure. In order to minimize the changes in the pH of the external solutions we renewed the sea water in contact with the cells frequently.

In the light-dark experiments, three light conditions were employed: the dim light of a laboratory room; the outside light on a porch with a southern exposure, but shaded so as to avoid direct sunlight; and finally the darkness of a room suitable for use as a darkroom in developing all but the fastest emulsions (there was a very small leakage

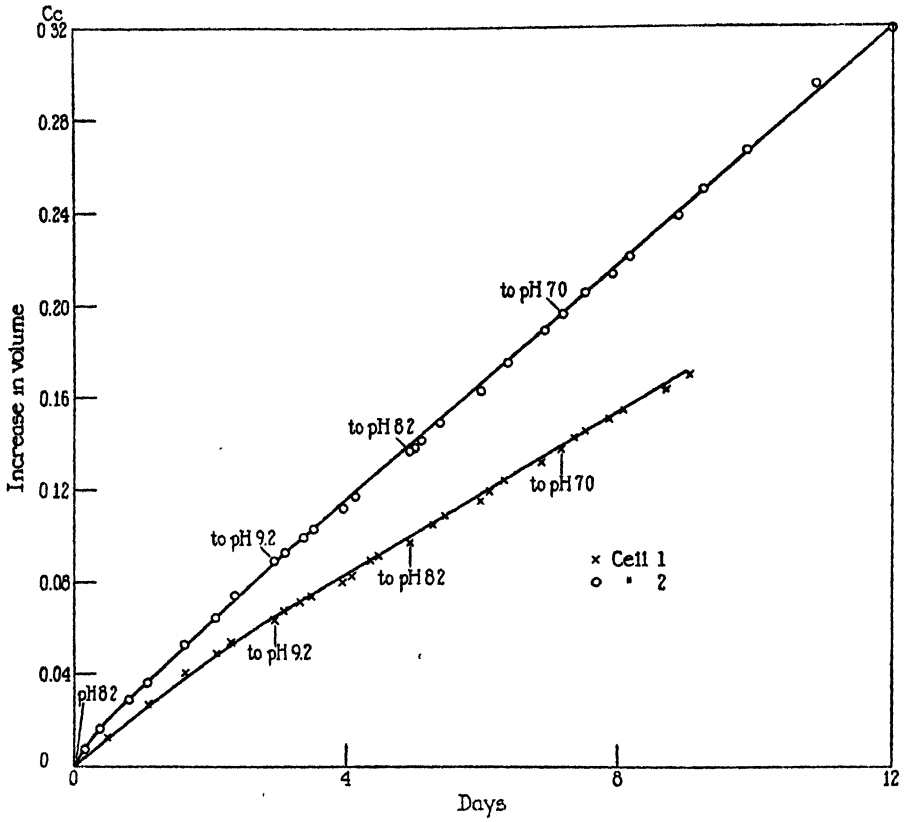

FIG. 1. Rate of increase of volume of sap of impaled *Halicystis* cells at different pH's of the sea water. Each curve represents the behavior of a single cell. In each case before the run started the cells had been seasoned in normal sea water for at least 5 days after impalement. Similar curves were obtained with other cells.

of light around the door). In the first case a Weston photronic foot-candle meter held near the cells gave a response of 32 foot-candles. In the second case on a cloudy day the response was 500 foot-candles and in the last case there was no response.

In all cases each bottle of cells was immersed to the neck in a tray through which passed a brisk flow of water from the salt water circulating system. Thus although the experiments were performed in three different parts of the Biological Station, the condi-

tions, except the lighting, were about the same. Except in the darkroom the lighting was normal; *i.e.*, the natural succession of light and darkness.

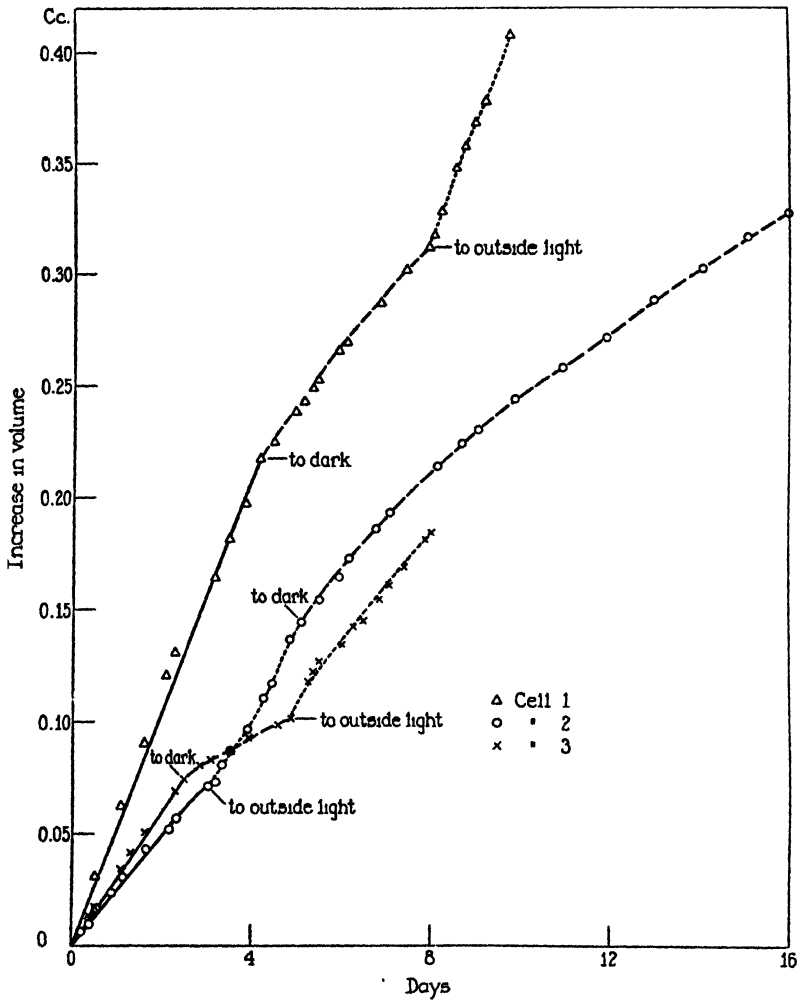

FIG. 2. Rate of increase of volume of sap of impaled *Halicystis* cells in light and darkness. Each curve represents the behavior of a single cell. The first part of the run in every case represents the behavior in dim light in the laboratory. The application of other light conditions is indicated directly on the curves. In each case before the run started the cells were seasoned in normal sea water for at least 5 days after impalement. The curves have been drawn free-hand to give an approximate fit.

As before, the rate of increase of volume of the sap was determined by measuring with a micrometer caliper the rise of liquid in a capillary of known radius. Then,

$$\Delta V = \pi r^2 h$$

where h is the capillary rise. On the basis of the previous experiments it was assumed that the electrolyte entered the cell at the same rate as the water and this was checked by determining the halide concentration of cells after exposure to different pH's and to different light conditions. As anticipated there was little or no decrease in halide concentration in any case.

The results of some of the experiments have been plotted in Figs. 1 and 2. In each case we have plotted the increase in volume of the sap against time, with notations at the points where pH or light conditions were changed. With the assumption that entrance takes place under "steady state" conditions (*i.e.* with the mole fractions of water and electrolytes in the sap remaining unchanged) the curves also show the behavior of electrolytes.[3]

DISCUSSION OF RESULTS

Before proceeding to discuss the results already obtained we shall review briefly some results previously obtained with impaled cells. It has been found with both *Valonia* and *Halicystis* that after a short initial rapid increase immediately after impalement,[4] the rate of uptake of water and electrolyte becomes practically linear. The rate is greater than in the intact cell, about 15 times as great in *Valonia* and 10 times as great in *Halicystis*. But the composition of the sap (except for a possible slight decrease in total osmotic concentration to a value approaching that of the sea water) remains unchanged.

It seems clear from experiments with impaled *Halicystis* cells in diluted sea waters that water moves much more rapidly through the protoplasm than does electrolyte: hence any increase in the osmotic pressure of the sap due to the entrance of electrolyte is quickly abolished by the entrance of water.

Now we suppose that all ionic species found in the sap have moved chiefly as molecules rather than as ions, in the non-aqueous protoplasmic

[3] We assume provisionally that not only does the total electrolyte concentration of the sap remain constant, but that the proportions of all ions in the sap remain unchanged. In the experiments with impaled *Valonia*, the concentration of potassium, for example, according to available analyses did not decrease in the impaled cells, and as the halide concentration also remained approximately constant it was assumed that the sodium concentration had not changed. In the case of impaled *Halicystis*, only halide analyses are available but there is little reason to suppose that there would be any marked change in the sap composition.

[4] Provisionally we assume that water enters at a greater rate than electrolyte, resulting in the adjustment of the osmotic pressure of the sap either nearer to or possibly to equality with that of the sea water.

surface layers. That is to say ionic movement in the protoplasmic surface is considered to be negligibly small, and since the protoplasmic surface is thin we can apply for the rate the equation

$$\frac{d[M]}{dt} = D^{MX_1}\{[MX_1]_{epo} - [MX_1]_{epi}\} \quad (1)$$

$$= D^{MX_1}\{S_{eop}[MX_1]_{eop} - S_{eip}[MX_1]_{eip}\} \quad (2)$$

here M is any monovalent cation, such as Na, K, or NH_4, and X_1 is either an organic anion derived from a weak acid HX elaborated by the protoplasm or an inorganic anion derived from the sea water. D^{MX_1} is a permeability constant analogous to the diffusion constant, S is the partition coefficient. *eop* and *epo* are a pair of adjacent regions in unstirred layers of sea water and protoplasm respectively, where all species are in equilibrium across the interface, the distribution being determined by the partition coefficient S (S = concentration in the non-aqueous protoplasmic surface layer ÷ concentration in the adjoining aqueous phase); the subscripts *o* and *i* refer to the outside and inside protoplasmic surfaces respectively.

In this derivation it is supposed for simplicity that MX_1 is a single species; if several are involved, as may well be the case, the situation will be more complicated.

Now we suppose that when the cell is impaled, for a particular set of conditions (lighting, temperature, pH of sea water, etc.), all the terms of equation (2) remain reasonably constant and hence the rate of uptake of electrolyte is linear. But in order to account for the slower rate of uptake in intact cells without any change in the sap concentration we have suggested that the partition and diffusion coefficients in such cells may be different. After the cellulose wall has been stretched to the very limited extent possible, water cannot enter the cell until new cellulose is formed and the area of the cellulose wall is increased so as to inclose more space. This is a slow process. Hence it seems possible that when the osmotic pressure of the sap increases through electrolyte entrance without the possibility of water entering immediately, water may be withdrawn from the non-aqueous layers of the protoplasm, with the result that both the diffusion and partition coefficients decrease. The net effect is to prevent the osmotic concentration of the sap from rising to a much greater value than that of the sea water.

Let us now consider results of the present experiments with impaled *Halicystis* in the light of these considerations.

As Fig. 1 clearly shows, changing the external pH between 7.0 and 9.2 has little or no effect on the rate of volume increase, and therefore of

uptake of electrolyte. The wave-like form of the curves associated with daylight and darkness, which was demonstrated in a previous paper,[5] is not very obvious in the present curves owing to the small scale used, and in any case, since each exposure to sea water of one pH lasted 2 days or more, when we compare the effects of different pH's the effects of the natural succession of daylight and darkness may be considered as equal in each case, and need not be considered especially.

Changes in pH cannot be expected to have any effect on either D or S, or the gradient term $[MX_1]_{eop}$ if X_1 is the anion of a strong electrolyte. For example, in the case of *Halicystis*, where most of the electrolyte in the cell is sodium chloride, M may be sodium and if X_1 is chloride ion, we have

$$[\mathrm{NaCl}]_{eop} = [\mathrm{NaCl}]_{op} = [\mathrm{NaCl}]_o$$

which is proportional to $[\mathrm{Na}]_o\ [\mathrm{Cl}]_o$. Here *op* is an unstirred layer in the sea water adjacent to a similar unstirred layer, *po*, in the protoplasm (the thin regions in these layers, where the solutes are in equilibrium across the interface, are *epo* and *eop*), and *o* refers to the main mass of sea water.[6] Obviously none of these values will be affected by changes in the pH of the sea water.

But if X_1 is the anion of a weak acid elaborated by the protoplasm the rate of entrance of sodium might be increased by increasing the pH of the sea water, but this would not be inevitable. Thus an increase in rate, such as we observe for the entrance of ammonia into *Valonia* when the external pH was raised,[7] may be good evidence in favor of the view that a reaction with a weak acid precedes the entrance of a base, but the failure of the rate to respond to pH changes is not definite evidence against this mechanism.

The amount of MX which can be formed depends on the amount of HX which is available and if at one pH practically all the HX has been converted to MX, raising the external pH may have only a slight effect in increasing the amount of MX.[5]

[5] Jacques, A. G., *J. Gen. Physiol.*, 1938–39, **22**, 757.

[6] In this we assume that there is no appreciable gradient of concentration of [Na] [Cl] or [NaCl] between the main mass of the sea water and the layer of sea water adjacent to the protoplasm since all these constituents are abundant in the sea water and also because their removal by diffusion through the protoplasm must be very slow compared with the rate at which they can be renewed by diffusion from outside. However, as pointed out previously, $[\mathrm{Na}]_o\ [\mathrm{Cl}]_o < [\mathrm{Na}]_i\ [\mathrm{Cl}]_i$ (where *i* refers to the sap) in the case of *Halicystis*. Hence if sodium is to enter as NaCl it will be necessary for the cell to supply energy.

[7] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1935, **21**, 125.

As we have seen in former papers, when X_1 is the anion of a weak acid HX elaborated by the protoplasm, equation (2) with certain assumptions assumes the form

$$\frac{dM}{dt} = k_{coll}\, D^{MX}\{[M]_o[\mathrm{OH}]_o[\mathrm{H}X]_{epo} - [M]_s[\mathrm{OH}]_s[\mathrm{H}X]_{epi}\}$$

where $k_{coll.}$[8] is a collection of constants including activity and partition coefficients (the corresponding constants are assumed to be equal in sap and sea water). The actual values in every case are unknown. Now if $[\mathrm{OH}]_o$ (or $(\mathrm{OH})_o$, which is the pH) is increased we should expect the rate to increase provided that $[\mathrm{H}X]_{epo}$ does not simultaneously decrease in the same ratio.

For convenience we assume that HX is being elaborated at a uniform rate throughout the non-aqueous protoplasm and the diagram below shows schematically how it and its ion X may be distributed in the system.

Sea water (*o*)	*eop*	*epo*	*epi*	*eip*	*sap*
$[\mathrm{H}X]_o$ ←	$[\mathrm{H}X]_{eop}$ ←	$[\mathrm{H}X]_{epo}$ ←	$[\mathrm{H}X]_{epi}$ ←	$[\mathrm{H}X]_{eip}$ →	$[\mathrm{H}X]_s$
↓↑	↓↑			↑↓	↑↓
$[\mathrm{Na}X]_o$ ←	$[\mathrm{Na}X]_{eop}$ →	$[\mathrm{Na}X]_{epo}$ →	$[\mathrm{Na}X]_{epi}$ →	$[\mathrm{Na}X]_{eip}$ →	$[\mathrm{Na}X]_s$

Now we may suppose that after a certain time (which may be very short) during which conditions (external pH, temperature, etc.) have remained constant, a steady state is reached in which the losses of HX at each interface are just balanced by the gains so that $[\mathrm{H}X]_{epo}$ and $[\mathrm{H}X]_{epi}$ remain constant though not necessarily equal.[9] It should be noted that in the steady state there may be little or no movement of HX and NaX from the *epi* layer to the sap, for the reason that the sap is limited in volume and even in the impaled cell it is increasing slowly. Hence if the partition coefficients are large, as may well be the case, only a little of the HX and NaX in the *eip* layer will need to move into the sap to keep the concentration at equilibrium. On the other hand, the sea water is unlimited in volume and hence there will be a steady diffusion of HX and NaX from *eop* to *o*.

Granting that the rate of HX production remains constant it will be clear

[8] For the exact significance of $k_{coll.}$ which is not needed here reference may be made to a recent paper (Jacques, A. G., *J. Gen. Physiol.*, 1938–39, **22**, 147).

[9] This is the assumption we have made in former papers and in fact in the case of ammonia entrance we found it possible to obtain a "permeability constant" from an equation similar to (2) on the assumption that $[\mathrm{H}X]_{epi} \simeq 7[\mathrm{H}X]_{epo}$.

from the results which follow that the effect of pH change will depend largely on the pK′ value of HX.

This may be illustrated by putting sets of hypothetical figures into the scheme just given. For simplicity we have made the following assumptions:

(*a*) HX and NaX have equal diffusion constants in the protoplasm, and different (probably much greater) but equal diffusion coefficients in the aqueous phases.

(*b*) S^{HX} and S^{NaX} are high, say 1000, and equal.

(*c*) The pH's of the *eop* and *eip* layers are regulated by relatively high concentrations of other buffer salts than NaX so that in these layers the hydrolysis of NaX has little effect on the pH which is approximately equal to that of the bulk of the sea water and sap respectively. The pH of the sap is assumed to be 5.2 (a little low but suitable for purposes of illustration).

The numerical schemes in Table I are divided into three pairs and comparisons of rate are to be made only between members of a pair. In the first pair (A) the pK′ of HX is assumed to be 10.2, in the second pair (B) 5.2, and in the third pair (C) 8.2. The external pH's are assumed to be 8.2 and 9.2. We have no idea of the actual concentrations of HX and NaX but the concentration terms are assumed to be millimolar.

The figures have been chosen so that the total concentration of species containing X at the *epo-eop* interface is about 100 millimolar and at the *epi-eip* interface 101 millimolar. The relation between HX and NaX is taken to be that calculated by the Henderson-Hasselbalch equation.[10] The undissociated portion is called for convenience HX and the dissociated portion NaX.

Now in every case, as the figures are chosen, the gradient which causes HX to move from *epi* to *epo* is about 1 unit greater than the gradient causing the diffusion of NaX from *epo* to *epi*. Thus in a unit of time (the same for the members of each group, but different for different groups) the *epi* layer loses 1 millimole of X by diffusion, but the steady state is preserved if, as we assume, in the same unit of time 1 millimole of new HX is produced at *epi* by the protoplasm. At the *epo* layer, 1 millimole of X is gained by diffusion in the protoplasm and concurrently it gains 1 millimole by new manufacture. But the steady state is preserved by the diffusion of 2 millimoles to the sea water.

Assuming that the rate of diffusion of NaX is proportionate to NaX_{epo} –

[10] With very slight modification so as to avoid too many fractional values. Thus in the first set of figures given for *epo* the ratio of HX to NaX should be (at pH 8.2 when pK′ = 10.2) 100 to 1 not 99 to 1, but the difference is negligible.

NaX_{epi} these calculations show quite clearly that with a high pK′ value the changing pH of the sap may have an effect on the rate of entrance of NaX nearly equal to the change in OH activity.

TABLE I

Hypothetical concentrations of HX and NaX in the outside protoplasmic surface layer (*epo*) and the unstirred layer of sea water (*eop*) just outside this and in equilibrium with it: also in the inside protoplasmic surface layer (*epi*) and the unstirred layer of sap (*eip*) just inside this and in equilibrium with it.

The ratio $HX \div NaX$ depends on the pK′ and the pH; when these are equal the ratio becomes unity.

		Sea water (*o*)	*eop*	*epo*	*epi*	*eip*	Sap (*i*)
A pK′ 10.2	pH	8.2	8.2			5.2	5.2
	Concn. HX	≃0	0.099	99	101	0.0101	0.0101
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.001	1	0.00101	0.00000101	0.00000101
	pH	9.2	9.2			5.2	5.2
	Concn. HX	≃0	0.090	90	101	0.0101	0.0101
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.010	10	0.00101	0.00000101	0.00000101
B pK′ 5.2	pH	8.2	8.2			5.2	5.2
	Concn. HX	≃0	0.0001	0.1	50.5	0.0505	0.0505
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.0999	99.9	50.5	0.0505	0.0505
	pH	9.2	9.2			5.2	5.2
	Concn. HX	≃0	0.00001	0.01	50.5	0.0505	0.0505
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.09999	99.99	50.5	0.0505	0.0505
C pK′ 8.2	pH	8.2	8.2			5.2	5.2
	Concn. HX	≃0	0.0505	50	101	0.0101	0.0101
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.0505	50	0.101	0.000101	0.000101
	pH	9.2	9.2			5.2	5.2
	Concn. HX	≃0	0.010	10	101	0.0101	0.0101
			↓↑			↓↑	↓↑
	Concn. NaX	≃0	0.090	90	0.101	0.000101	0.000101

On the other hand when pK′ is low there may be little or no observable difference in rate. For example, with pK′ = 5.2 we find that at pH 8.2 the concentration gradient $NaX_{epo} - NaX_{epi} = 99.9 - 50.5 = 49.4$ and at pH 9.2 it is $99.99 - 50.5 = 49.49$. Hence the rate of entrance of sodium would be practically the same at pH 8.2 and 9.2.

If the pK′ is of the same order as the pH's of the sea water to which the cells are subjected there will be a definite change in the rate of Na*X* diffusion, but not nearly so great as the change in the OH activity. Hence even if the rate does not respond to pH changes it does not exclude the possibility that the Na enters the cell as Na*X*. However, H*X* is probably not one but several acids and we should expect that the rate of entrance of some molecules would respond to pH changes.

Turning to the effects of light on the rate of gain of water and electrolyte we may first compare the curves for cells 1 and 3 of Fig. 2 since both had the same treatment and the results in general were the same. After the preliminary "healing" period lasting up to 5 days (not shown in the figure) each cell was exposed a few days to dim laboratory light (inside daylight with the natural succession of day and night) during which period the volume of the sap increased steadily at a roughly linear rate.[11] Then each cell was removed to nearly total darkness and the rate changed more or less abruptly and as long as the darkness persisted was lower than before and roughly linear. Each cell was then removed to outside light (on a shaded porch). The rate increased more or less abruptly and became roughly linear and about equal to the rate observed at the start in dim light. There was no great difference between dim laboratory light and "daylight," but there was a definite difference between light and darkness, the rate of volume increase being greater in light.

Cell 2 was first transferred from dim laboratory light to outside light and in this case the rate increased slightly. Then it was removed to darkness and the rate fell rather slowly and eventually became roughly linear and somewhat smaller than the rate in dim light, and of course still smaller than the rate in outside light.

Summing up, the increase of volume (and presumably the entrance of electrolyte) tends to be a linear function of time both in light and in darkness, but proceeds more rapidly in light. Between dim light (normal light indoors) and outdoor light (on a shaded porch) there is little difference. In general the rate in light was about twice the rate in darkness.

Some figures showing the total increase during the experiments may be of interest. In the case of cell 2, the total gain in volume of sap in 12 days was about 0.27 cc. which was 117 per cent of the original volume. Cell 3 gained 0.19 cc. in 8 days or 59 per cent of the original volume. There is little question that the rate fell off continuously but slowly in the dark.

Since light increases photosynthesis it probably increases the pH in the

[11] Neglecting the slight deviation from the straight line associated with daylight and darkness (*cf.* Jacques, A. G., *J. Gen. Physiol.*, 1938–39, **22**, 757).

layer of sea water adjacent to the protoplasm by removing CO_2. However, as we have shown, the rates of entrance of water and of electrolytes do not respond to direct changes in the pH of the sea water so that we can scarcely explain the increased rate in the light as a pH effect. However, if light should cause the cell to manufacture H*X* more rapidly, more Na*X* could be produced irrespective of the relation of pK′ to the pH of the external solution. That is to say the rate of entrance of sodium as Na*X* could respond to changes in the H*X* concentration without necessarily responding to changes in the OH activity of the sea water.

We may compare this with the idea suggested by recent results with *Valonia* obtained with ammonia accumulation in light and darkness[12] and with changes in the external pH,[7] where the rate of entrance of ammonia is undoubtedly accelerated by increasing the external pH. It is also accelerated by light. Without going into details we may say that calculations in the ammonia paper indicated that the permeability constant in the dark was about 3 times that in the light, although owing to the higher pH in the light the amount of ammonia entering the cell per unit of time was greater. But as calculated the constants contained a factor *c* which referred to changes in the protoplasm and it was assumed that this was about 3 times greater in darkness than in light, and that it represents the concentration of the acid H*X* at the sap-protoplasm interface. It was also assumed that the H*X* concentration at the sea water-protoplasm interface (*b* in the previous paper) was also 3 times as great in the dark. Hence the decrease in rate in the dark due to the lower pH was in part offset by the increase in H*X* in the protoplasm. This might arise either from a more rapid production of H*X* in the dark, or from a slower rate of loss to the sea water.

In the case of *Halicystis*, if sodium enters as Na*X*, we must assume that the acid in question is at a higher concentration in the light.

SUMMARY

The rate of entrance of electrolyte and of water into impaled cells of *Halicystis Osterhoutii* is unaffected by raising the pH of the sea water to 9.2 or lowering it to 7.0. It is quite possible that sodium enters by combining with an organic acid H*X* produced by the protoplasm. If the pK′ of this acid is sufficiently low the change in external pH would not produce much effect on the rate of entrance of sodium.

The rate of entrance of electrolytes is affected by light. In normal light (*i.e.* natural succession of daylight and darkness) the rate is about twice as great as in darkness.

[12] Jacques, A. G., *J. Gen. Physiol.*, 1938–39, **22**, 501.

CALCULATIONS OF BIOELECTRIC POTENTIALS

V. Potentials in Halicystis

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Interest in *Halicystis* and in *Valonia* has been stimulated by discoveries of marked contrasts and striking similarities existing side by side.

Although originally regarded as similar and placed in the same genus they are now classified in separate orders: they show great differences in morphology and reproduction,[1] in composition of sap,[2] and in bioelectric behavior.[2] As examples of the last may be mentioned:

(1) The normal P.D. is outwardly directed (positive[3]) in *Halicystis*[4] (65 mv.),[5] but inwardly directed (negative) in *Valonia*[4] (10 mv.). When these cells are placed in their own sap so as to set up the chain

sap | protoplasm | sap

the P.D. is negative in *Valonia* (60–70 mv.)[6] and positive in *Halicystis* (40–50 mv.).[5] These P.D.'s must be due to conditions in the protoplasm, which are strikingly different in the two plants.

(2) Guaiacol[7] changes the P.D. of *Valonia* in a positive direction and that of *Halicystis*[7] in a negative direction. It decreases the apparent mobility of K^+ in *Valonia* but not in *Halicystis*.

(3) Lowering the pH of sea water changes the P.D. of *Halicystis* in a negative direction[5, 7] but has practically no effect on the P.D. of *Valonia*.

(4) Very low concentrations[8] of NH_3 produce drastic changes in the

[1] Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1930-31, **57**, 389.

[2] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967.

[3] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution; *i.e.*, it is outwardly directed.

[4] In this paper when *Valonia* is mentioned *V. macrophysa*, Kütz., is meant and *Halicystis* means *H. Osterhoutii*, Blinks and Blinks.

[5] Blinks, L. R., *J. Gen. Physiol.*, 1934–35, **18**, 409.

[6] Damon, E. B., *J. Gen. Physiol.*, 1931–32, **15**, 525.

[7] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13; 1937–38, **21**, 707.

[8] Blinks, L. R., *J. Gen. Physiol.*, 1933–34, **17**, 109. For the effects of flow of current see Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 633, 867.

P.D. of *Halicystis* (including reversal of the sign) but have little or no effect on *Valonia*.

Resemblances between the two plants are illustrated by their behavior toward alkali cations. The situation in *Halicystis* is illustrated in Table I. The P.D. in sea water is about 65 mv. positive.[9] We see that partial or complete replacement of Na^+ by K^+ or Rb^+ changes the P.D. in a negative[3] direction. This is also the case with *Valonia*, and the extent of the change is similar in both forms. Replacement of Na^+ by Li^+ produces no change, whence we conclude that the apparent mobility of Li (or u_{Li}) in the outer

TABLE I

Changes in P.D. Caused by Substituting Various Chlorides (MCl) for Sea Water (S.W.)

The number of observations is given in parenthesis. Sea water was positive in the external circuit in all cases where a difference of P.D. existed.

MCl	Sea water to 0.6 M MCl, 1 part + S.W., 1 part	u	Sea water to S.W. containing 0.52 M MCl	u	Sea water to S.W. containing 0.52 M MCl, 1 part + mannite solution, 1 part	u	Average for u
	mv.		mv.		mv.		
KCl	48.0 ± 2.1 (11)	$u_K = 16$	63.0 ± 1.3 (14)	$u_K = 18$	51.0 ± 3.3 (4)	$u_K = 19$	$u_K = 17.7$
RbCl	48.4 ± 3.2 (15)	$u_{Rb} = 16$	62.6 ± 1.2 (7)	$u_{Rb} = 18$	46.5 ± 2.9 (7)	$u_{Rb} = 15$	$u_{Rb} = 16.3$
CsCl	22.2 ± 1.6 (12)	$u_{Cs} = 4.4$	48.8 ± 2.2 (3)	$u_{Cs} = 9.5$	35.8 ± 3.0 (4)	$u_{Cs} = 8.5$	$u_{Cs} = 7.5$
LiCl	Zero (5)	$u_{Li} = 0.2$	—	—	—	—	$u_{Li} = 0.2$

Note. Sea water to (0.4 M $MgCl_2$ 1 part plus sea water 1 part) gave no change of P.D. (5 observations) whence $u_{Mg} = 1.9$.

Sea water to (0.4 M $CaCl_2$ 1 part plus sea water 1 part) gave a change of 56.4 ± 2.0 mv. (5 observations) in a negative direction (but no calculation can be made).

protoplasmic surface is equal to that of Na (or u_{Na}). The latter has been found to be 0.2 in *Valonia* and the concentration effect[10] shows that it is about the same in *Halicystis*.

We also see differences in experiments with the alkali metals. In *Halicystis* u_{Cs} is higher than v_{Cl} and higher than u_{Na}, but in *Valonia* u_{Cs} is lower than v_{Cl} and about equal to u_{Na}.[11]

[9] Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 633.

[10] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445. Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 75; *J. Gen. Physiol.*, 1937–38, **21**, 707. See footnote 19.

[11] Damon, E. B., *J. Gen. Physiol.*, 1938–39, **22**, 819.

On replacing sea water by 0.4 M $MgCl_2$ plus an equal volume of sea water we find no change in P.D. The calculation shows that if we put $u_{Mg} = 1.9$ we get 0.02 mv. with sea water positive in the external circuit but if we put $u_{Mg} = 1.8$ we get 0.29 mv. with sea water negative. If we assign still lower values to u_{Mg} the calculated value for sea water becomes correspondingly more negative. We therefore take u_{Mg} as 1.9.

The measurements were made on *Halicystis Osterhoutii*,[12] Blinks and Blinks, using the technique described in former papers.[13] Some of the measurements were made by W. C. Owens, using a D'Arsonval galvanometer in place of the string galvanometer employed in previous work.

No evidence of injury was observed in these experiments.

The temperature was 20 to 25°C.

The RbCl and CsCl were obtained from Theodor Schuchardt, the remaining salts from Kahlbaum.

In column II of Table I are shown the changes[14] in P.D. produced by replacing Bermuda sea water by a solution made by mixing equal parts of Bermuda sea water and 0.6 M solution of a chloride. The mixture was brought to the pH of sea water (8.2); this applies to all the solutions used in this investigation unless otherwise stated.

Column IV gives the changes in P.D. produced by replacing the sea water by an artificial sea water containing 0.52 M of a chloride in place of the 0.488 M NaCl found in Bermuda sea water.[15]

Column VI gives the changes observed when the solutions of column IV were diluted with an equal volume of a solution containing 1.1 M mannite[16] + 0.012 M KCl + 0.02 M $CaCl_2$. The note appended to the table gives the results obtained by replacing sea water by a mixture[17] of 0.4 M $MgCl_2$ or of 0.4 M $CaCl_2$ with an equal volume of sea water (both at pH 7.8).

The calculations of the apparent mobilities u_K, u_{Rb}, u_{Cs}, and u_{Li} were made as in former papers[18] by means of Henderson's equation, putting $v_{Cl} = 1$, and disregarding[10]

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[12] In the experiments here described the P.D. at the start (in sea water) was normal in all cases.

[13] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13. Regarding the amplifier see Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541.

[14] The P.D. is determined as in Damon's experiments (Damon, E. B., *J. Gen. Physiol.*, 1931–32, **15**, 525; 1932–33, **16**, 375; 1937–38, **21**, 383) by taking the first maximum of the P.D. time curve.

[15] For a time curve of the changes due to sea water containing 0.52 M KCl see Blinks, L. R., *J. Gen. Physiol.*, 1932–33, **16**, 147, and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 631, 707. The time required to reach the maximum was about 30 seconds for the cases dealt with in columns IV and VI and about 3 or 4 times as long for those dealt with in column II.

[16] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 75.

[17] These mixtures were approximately isotonic with the sea water.

[18] *Cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

Mg^{++} and Ca^{++}. The value of u_{Na} was taken as 0.2 as in *Valonia*[19] and the value of u_K in each column was taken as that given in the upper part of the column. Thus in column IV it was found by trial that $u_K = 18$ (putting $v_{Cl} = 1$). We then have for RbCl the following

$$U_I = u_{Rb} C_{Rb} = u_{Rb} (0.52)$$
$$V_I = v_{Cl} C_{Cl} \text{ is taken for convenience as } 0.5$$
$$U_{II} = u_{Na} C_{Na} + u_K C_K = 0.2(0.488) + 18(0.012) = 0.314$$
$$V_{II} = v_{Cl} C_{Cl} = 1(0.5)$$

We find by trial that $u_{Rb} = 18$.

The calculations have been made (as for *Valonia* in former papers) on the assumption that the partition coefficient S (S = concentration in the external non-aqueous protoplasmic surface ÷ concentration in the external solution) is the same for all the salts. This is probably not correct since it does not hold for *Nitella*[20] but it is the best that can be done under the circumstances since the mobilities of K^+, Rb^+, Cs^+, and Li^+ cannot very well be obtained by measuring the concentration effect. The difficulty may be illustrated as follows. In an artificial sea water containing KCl in place of NaCl (which will be called "K-sea water" for convenience) the P.D. changes first in a negative and then in a positive direction.[21] A similar situation exists in *Valonia*.[22] It is customary in these cases to take the apex of the curve as indicating the full effect of KCl but if no penetration took place a higher value might be obtained.

If we wait until the apex of the curve is reached and then substitute for "K-sea water" a solution containing "K-sea water" + 1 part of isotonic mannite solution[16] the curve begins to fall but as it would also fall in case no change of solution were made we cannot measure the concentration effect. This difficulty is also found with RbCl and CsCl.

It is of interest to note that the apparent mobility of Cs^+ in the outer protoplasmic surface is lower than that of K^+ whereas in water it is higher. In general the values of the mobilities do not agree with those found in water. To what extent this is due to solvation or to the formation of complexes is uncertain.[23]

It is also of interest that the values of the apparent mobilities in the outer protoplasmic surface agree so well when calculated from different combinations.

Thus far the behavior of *Halicystis*, as summarized in Table I, agrees very well with that of *Valonia*,[11] the greatest difference being found with CsCl which with *Valonia* acts very much like NaCl but with *Halicystis* behaves as though u_{Cs} were greater than u_{Na} and v_{Cl}.

[19] According to Damon and Osterhout (footnote 10) the change in P.D. on replacing sea water by sea water plus an equal volume of isotonic glycerol solution amounts to 11.6 mv. With *Halicystis* the same dilution, using mannite solution (footnote 15) produces a change of 11.8 ± 0.1 (27 observations).

[20] Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312.

[21] Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 632 (Fig. 1).

[22] Damon, E. B., *J. Gen. Physiol.*, 1932–33, **16**, 375; 1937–38, **21**, 383.

[23] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

In *Valonia* u_{NH_4} is greater than u_K or u_{Rb}:[11] in *Halicystis* the value of u_{NH_4} is uncertain because of the disturbing action of NH_3. If we replace sea water by 0.6 M NH_4Cl plus an equal volume of sea water the concentration of NH_3 is great enough even at low pH to produce the reversal of sign described by Blinks.[8] But according to Blinks at pH 5 low concentrations of NH_4Cl may act very much like KCl.[8]

With respect to $MgCl_2$ the two plants act similarly but with $CaCl_2$ a very different situation develops. Replacement of sea water by 0.4 M $CaCl_2$ plus an equal volume of sea water makes a change in the negative direction of 56.4 mv. But, as Blinks has shown, a similar change is produced by absence of $CaCl_2$.[24] Hence it is evident that the protoplasmic surface undergoes alteration and that Henderson's equation is not obeyed. In *Valonia* absence of calcium has little or no effect.[25]

SUMMARY

Interest in the study of *Halicystis* and of *Valonia* has been stimulated by discoveries of marked contrasts and striking similarities existing side by side. This is illustrated by new experiments with the alkali metals and alkaline earths.

In *Halicystis* the apparent mobilities of K^+, Rb^+, Cs^+, and Li^+ (calculated by means of Henderson's equation from changes in P.D. produced by replacing sea water by a mixture of equal parts of sea water and 0.6 M of various chlorides) are as follows. $u_K = 16$, $u_{Rb} = 16$, $u_{Cs} = 4.4$, and $u_{Li} = 0.2$; u_{Na} is taken as 0.2. These values resemble those in *Valonia* except that in the latter u_{Cs} is about 0.2.

No calculation is made for u_{NH_4} because in these experiments even at low pH so much NH_3 is present that the sign of the P.D. may reverse. This does not happen with *Valonia*. According to Blinks, NH_4^+ at pH 5 in low concentrations acts like K^+.

The calculation gives $u_{Mg} = 1.9$ which is similar to the value found for *Valonia*.

No calculation can be made for $CaCl_2$ since it produces protoplasmic alterations and in consequence Henderson's equation does not apply. This differs from *Valonia*.

Evidently these plants agree closely in some aspects of electrical behavior but differ widely in others.

[24] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 223. Blinks, L. R., Rhodes, R. D., and McCallum, G. A., *Proc. Nat. Acad. Sc.*, 1935, **21**, 123.

[25] Transferring from sea water to 0.6 M NaCl has little or no effect on the P.D. of *Valonia*. *Cf.* footnote 23.

INCREASE IN BACTERIOPHAGE AND GELATINASE CONCENTRATION IN CULTURES OF BACILLUS MEGATHERIUM

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The increase in phage or virus concentration in the presence of living cells is formally analogous to the formation of enzymes by cells (1-5 and 54) or from their precursors (6), as well as to the growth of cultures of bacteria (40). There is an essential difference between the growth of cells and the formation of enzymes in that cells can use energy to synthesize themselves from simpler compounds whereas, so far as is known, enzymes cannot. It is not yet known whether phage or viruses possess this power of synthesis. Until this knowledge is obtained it does not seem possible to decide as to which of the two analogies has a real physical basis and which is merely formal. In the meantime it appears to the writer that the much more simple analogy with enzyme formation has predicted the facts better than the analogy with cell growth. Thus, some viruses and phages, at least, are proteins (6-9) and may be isolated by the methods of enzyme chemistry; they may be inactivated and reactivated (10-13); they have no measurable respiration (14). Pepsin and trypsin are immunologically distinct from their precursors (15-16), just as phage and virus are immunologically distinct from their host cells.

Pepsin and trypsin are formed by inoculation (28, 45) of solutions of their precursors with the active enzyme, just as phage or virus is formed after inoculation of the host cells with phage or virus. Some cells ("lysogenic" bacteria, virus "carriers") always produce phage or virus just as cells in general always produce "normal" enzymes, and it has been suggested (1, 3, 5) that phage is a normal enzyme of certain cells which is injurious to others. Such lysogenic strains, however, may be produced in the laboratory by growing resistant strains in the presence of phage (22, 40). This result renders less probable the assumption that phage is a normal product of the naturally occurring lysogenic strain. New "adaptive" enzymes may be formed by cells when grown in the presence of different substrates (17-21,

46–48). The problem of the production of new, active, substances is therefore encountered in relation to enzymes as well as to phage or virus.

Both phage and enzymes are usually produced only by growing cells, but under special conditions they may be produced without any cell growth (17–18, 29). Krueger (30–31) has shown that this ability to produce phage without growth is possessed only by cells which have just begun to divide. Hegarty (48) has found that it is precisely these "young" cells which can produce new enzymes. The production of virus in embryonic tissue (49) suggests a similar relation between the condition of the host cell and virus production.

The curves for the rate of formation of phage (23) and of enzymes (24–27) by cells or from their precursors (28, 45) are similar and more or less autocatalytic in character.

It is evident from the foregoing that a formal analogy exists between the production of phage and the formation of enzymes. If this analogy has any real physical significance the simultaneous formation of phage and of an extracellular enzyme in the same culture should follow very similar curves. In order to test this prediction the rate of formation of phage and of a gelatinase by lysogenic and "sensitive" strains of *B. megatherium* has been determined under a variety of conditions. In general the curves for the increase of phage and of gelatinase are very similar. There is a rapid increase in phage and enzyme concentration during the growth stage of the bacteria. The increase in all these quantities is logarithmic (autocatalytic); *i.e.*, the amount formed is proportional to the amount already present. The concentration of phage and gelatinase reaches a maximum and then decreases. The decrease may or may not be accompanied by a decrease in the number of cells depending upon the conditions and strain of bacteria used. The production of phage may be separated from that of gelatinase by varying the calcium concentration. In the presence of high calcium concentration little phage is produced while the gelatinase increases to a measurable extent, but less than it does in low calcium concentration. In the absence of calcium no phage is produced and the production of gelatinase is decreased.

If resting cells are used no increase in phage or gelatinase can be detected without cell growth. If rapidly growing cells are used, however, a narrow range of acidity exists near pH 5.5 in which no further cell growth occurs but a large increase in phage may be obtained. Under proper conditions it is therefore possible to separate phage production from cell growth as may be done with enzyme formation.

EXPERIMENTAL RESULTS

1. Megatherium Culture and Phage.—The culture of *megatherium* (899 T) used was isolated originally by De Jong and further studied by De Jong (5), Gratia (32-35), Wollman (36), and others. The writer is indebted to Professor Gratia for supplying him with the various strains of the bacteria and also with the phage. The organism forms two types of cultures: (1) a smooth strain (899 S) which does not spore and, (2) a spore-forming strain (899 R). Both strains produce a bacteriophage which causes lysis of a sensitive strain (Meg. 36 S) but does not cause lysis of the lysogenic culture 899 which produces it.

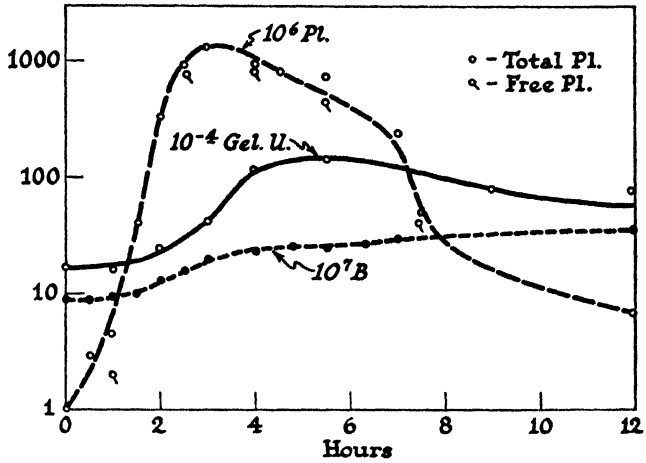

FIG. 1. Increase in plaque count, number of bacteria, and gelatinase concentration during growth of resistant strain, 899 T. Yeast extract media shaken at 35°C.; inoculated from 18 hour 30°C. Blake bottle culture.

Two types of phage are formed (34), one of which causes complete lysis of Meg. 36. This "C" phage was used in the present work. A second type of phage causes only partial lysis of Meg. 36 (Phage T).

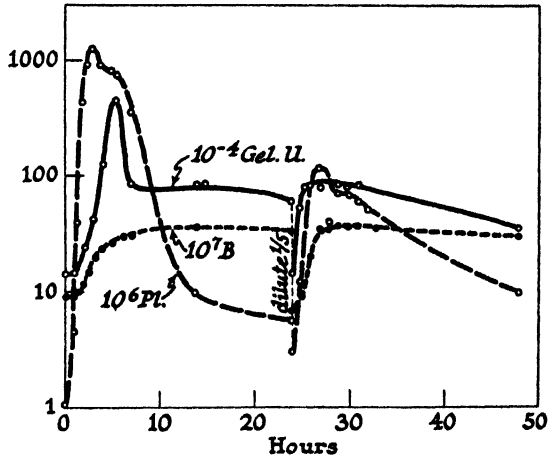

FIG. 2. Increase in plaque count, number of bacteria, and gelatinase concentration during growth of resistant strain 899 T. After 24 hours 10 cc. of culture added to 40 cc. fresh yeast extract media, shaken at 35°C.

2. Increase in Number of Bacteria, Bacteriophage, and Gelatinase in

Growing Lysogenic Cultures of B. megatherium (899 T) in Yeast Extract Media at 35°C.—The results of an experiment in which the bacteria concentration, the plaque count, and the gelatinase activity were determined are shown in Fig. 1.

There is a lag period of about ½ hour in which no change is detectable. The cell concentration then increases continuously while the phage concentration and gelatinase concentration increase much more rapidly than the cells at first, reach a maximum, and then decrease. The curves are all logarithmic during the stage of rapid increase. The cell concentration remains constant after about 14 hours. There is no demonstrable lysis. The maximum number of plaques per ml. is about 1000×10^6 or about 2–3 times that of the maximum bacteria concentration 40×10^7. The plaque count in the filtrate is nearly the same as that of the suspension. The maximum gelatinase concentration is $100\text{-}200 \times 10^{-4}$ gelatin units per ml. The relation of gelatinase production to cell growth is the same as that found by Kocholaty, Weil, and Smith (25) with *Cl. histolyticum.*

3. Increase in Phage, Bacteria, and Gelatinase in Successive Subcultures.—The results of an experiment in which 10 ml. of culture was added to 40 ml. of fresh yeast extract after 24 hours are shown in Fig. 2. The general slope and relation of the curves remain the same although there is considerable variation in the maximum gelatinase concentration and the maximum plaque count obtained. This experiment was carried through five subcultures without any significant change in the results. Repetition of the experiment gave the same general result. In some cases the maximum gelatinase concentration was much higher and was reached later than in the experiment reported. The decrease in the phage and gelatinase is caused by the growth of the culture and is not due to simple inactivation since filtrates retain their activity under the same conditions.

Increase in Phage, Bacteria, and Gelatinase in Cultures of the Sensitive Strain (B. megatherium 36)

1. Lower Phage Concentration.—The changes in phage and cell concentration of a culture of the susceptible strain inoculated with phage depend upon the relative initial concentration of cells and phage. If cultures containing more than 10^7 bacteria per ml. of *megatherium* 36 are inoculated with lower concentration of phage so as to give 10^3 or less plaques per ml. the results are very similar to those obtained with the resistant strain (Fig. 3). The phage concentration reaches a maximum during the rapid growth of the bacteria and then decreases. The relative concentration of phage to bacteria at this point is below the critical value

and therefore no lysis occurs. The maximum gelatinase concentration in this experiment was reached later. The oxygen consumption per minute was determined in a Warburg respirometer. It increases at first in proportion to the increase in bacteria and then decreases at about the time the plaque count decreases. A similar relation between oxygen consumption and virus production was found by Zinsser and Schoenback (37).

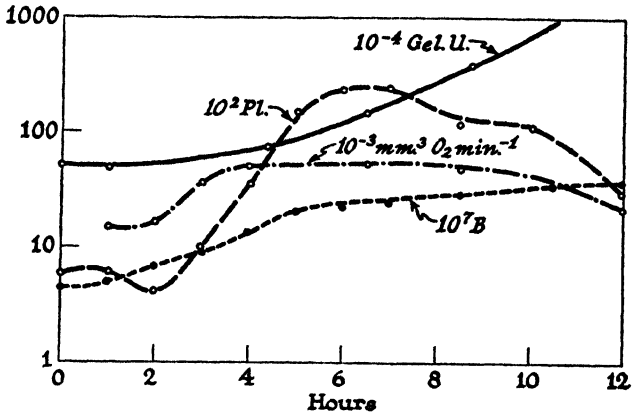

FIG. 3. Increase in plaque count, number of bacteria, and gelatinase concentration and oxygen consumption per minute of susceptible strain, *megatherium* 36 sensitive. Inoculated with low concentration phage "C;" yeast extract media, shaken at 35°C.

2. High Phage Concentration.—If cultures of the sensitive strain containing less than 10^8 bacteria per ml. are inoculated with high phage concentration so that the initial plaque count is 10^6 per ml. or more lysis occurs. Under these conditions the results are more irregular. If the ratio of phage to bacteria is high the critical ratio necessary for lysis is soon reached and lysis occurs rapidly and is nearly complete. As the ratio of phage to bacteria is decreased lysis is delayed and is less complete and a resistant strain of the organism develops in the culture (Fig. 4). This resistant strain also produces phage but does not undergo lysis. Occasionally a second increase in growth rate occurs and is accompanied by a second increase in phage

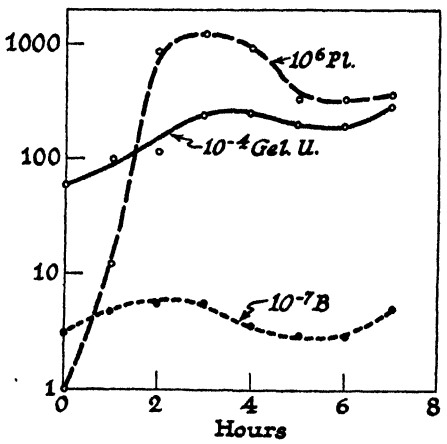

FIG. 4. Increase in plaque count, number of bacteria, and gelatinase concentration of susceptible strain, *megatherium* 36 sensitive. Inoculated with high concentration of phage "C;" yeast extract media, shaken at 35°C.

(Fig. 5). If subcultures are made after 24 hour intervals the curves are similar but the maximum phage and bacteria concentration obtained vary depending upon the relative amounts present at the time the subculture was made (Fig. 6).

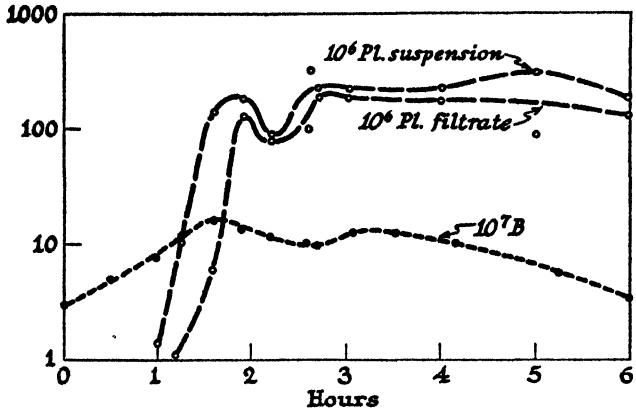

FIG. 5. Increase in plaque count and bacteria concentration of *megatherium* 36 sensitive in yeast extract media shaken at 35°C.

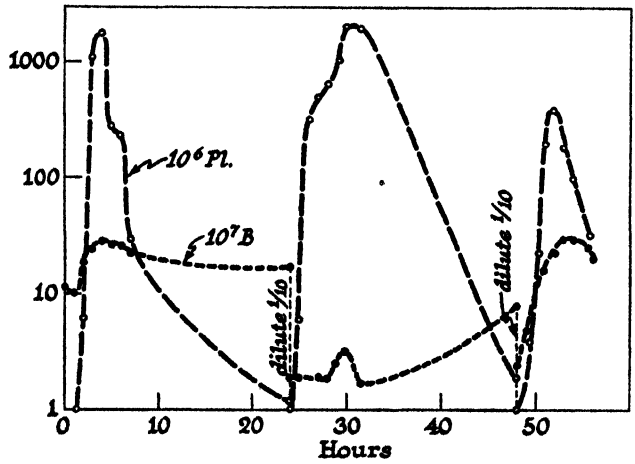

FIG. 6. Increase in plaque count and bacteria concentration in successive cultures of *megatherium* 36 sensitive in yeast extract shaken at 35°C. Subculture made at 24 hour intervals by adding 10 cc. of old culture to 100 cc. of fresh yeast extract.

If the original culture is shaken for 48 hours the resistant strain grows during the second day and this is accompanied by a second increase in phage and gelatinase (Fig. 7).

If a subculture is made from the resistant culture phage and gelatinase are again produced during the growth of the culture but no lysis occurs. The culture now behaves (35) like the original lysogenic strain 899 T. It produces phage but does not undergo lysis. The phage and gelatinase concentration were unusually high in this experiment and it may be noted that nearly as much phage was formed by the resistant subculture as in the original sensitive culture.

Free and Total Phage and Gelatinase.—The increase in free and total

phage during growth of the culture is shown in Fig. 8. 70-80 per cent of the phage is free as Gratia states (33). This ratio remains nearly constant during the growth of the culture. In a few experiments (Fig. 5)

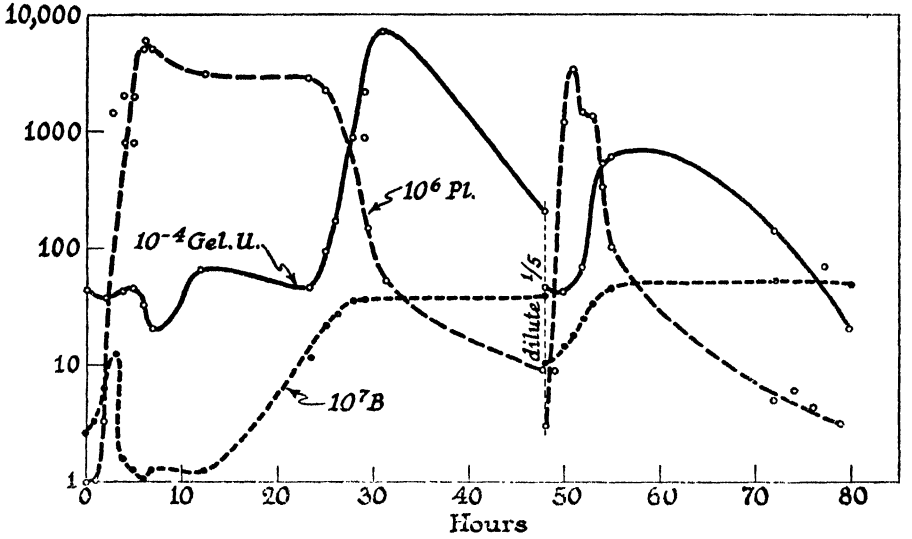

FIG. 7. Increase in plaque count, bacteria concentration, and gelatinase concentration of culture *megatherium* 36 sensitive in yeast extract media shaken at 35°C. Subculture made after 48 hours when resistant strain was developed.

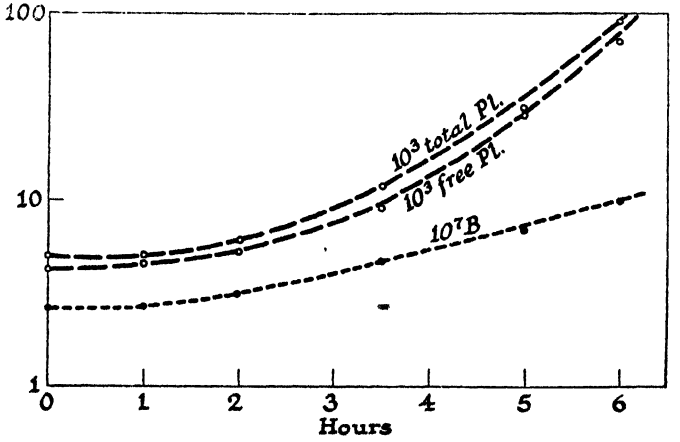

FIG. 8. Increase in plaque count in suspension (total) and in filtrate (free) in the presence of growing cultures of *megatherium* 36 sensitive. Yeast extract media shaken at 26°C.

the percentage of free phage was lower during the first part of the curve. There is some indication that the per cent of free phage increases at about the time lysis commences but the concentrations are changing very rapidly with time at this stage of the reaction and it is difficult to be sure that the free and total phage counts on a sample are really comparable owing to the necessary difference in handling the sample. It seems probable that this difficulty accounts for the discrepancy between Wollman's (36) and Gratia's (33) results. No further change in the amount of free phage occurs on standing. Most of the phage combined with the cells may be removed by repeated washing but about one-quarter of the amount remains fixed. It must be noted that these figures are all "infective units" and may not represent actual quantity of phage. It is quite possible that much larger quantities of phage are combined with the cells than these figures indicate since one infected cell will show only one plaque no matter how much phage is in or on the cell.

The gelatinase activity of the filtrate is the same as that of the whole suspension. Washed cells have no demonstrable gelatinase activity. Similar results have been obtained by Kocholaty, Weil, and Smith (25) with *Cl. histolyticum.*

Increase in Total or in Combined Phage.—The preceding experiment shows that most of the phage in these cultures is free. This fact renders it possible to determine whether the increase in phage depends on the total phage concentration or on the quantity of combined phage. With most cultures such as *Staphylococcus* this experiment cannot be done since practically all the phage is combined with the cells.

In order to determine whether the increase in phage is proportional to the total or to the combined phage a culture was inoculated with phage and shaken until the cell and phage count was beginning to increase. 5 ml. aliquots of the culture were then centrifuged and one set of aliquots stirred again and shaken at 35°C. The supernatant solution containing the free phage was removed from the other aliquots and the supernatant of a similar culture but which contained no phage, added to the precipitate. The precipitate was then stirred and the suspension shaken. The results of this experiment are shown in Table I. The figures are the average of four tubes. They show that the entire suspension contained 1300 plaques per ml. while a suspension of the precipitate contained only 300 plaques per ml. After 2 hours shaking both suspensions contained about 18,000 plaques per ml. Thus the same amount of phage was produced by the precipitate alone as by the whole suspension although the latter contained originally 4 times as many infective units. The quantity of phage produced is there-

fore determined by the combined phage only. As in the previous experiment there is the possibility that the actual quantity of phage in the precipitate is much larger than that indicated by the plaque count in which case this experiment is inconclusive.

Effect of Phosphate, Oxalate, or Excess Calcium on Phage or Gelatinase Production.—Bordet (4) and Gratia (35) have found that calcium is necessary for the production of phage. Merrill and Clark (38) found that

TABLE I

Formation of Phage by Whole Culture and by Precipitate Alone

100 ml. peptone plus bacteria from 18 hr. Blake bottle plus 0.01 ml filtered phage. Shake at 25°C.			100 ml peptone plus bacteria from 18 hr. suspension No phage. Shake at 25°C.
I			II
Hrs. at 25°C.	Bacteria per ml.	Plaques per ml	Bacteria per ml.
0	0.37×10^8	200	0.40×10^8
1.5	0.52		0.55
3.0	0.80		0.83
4.0	0.92	1,600	0.95

Eight 5.0 ml. aliquots of No. I centrifuged 10 minutes

Aliquots No. 1, 2, 3, 4 resuspended in supernatant. Transferred to flasks and shaken at 25°C.			Aliquots No. 5, 6, 7, 8, supernatant removed and replaced by supernatant from No. II. Precipitate stirred and suspension shaken at 25°C.	
Hrs. shaken	Bacteria per ml.	Plaques per ml.	Bacteria per ml.	Plaques per ml.
	Average of 4 tubes		Average of 4 tubes	
0	1.06 ± 0.05	$1,300 \pm 100$	1.08 ± 0.05	300 ± 50
1	1.34 ± 0.05	700 ± 100	1.40 ± 0.05	200 ± 50
2	1.84 ± 0.05	$17,300 \pm 300$	1.70 ± 0.10	$19,600 \pm 300$
Increase in plaques per ml.		16,000		19,300
Per cent increase in plaques per ml.		1,000		6,000

proteus would not produce proteinase without calcium. Similar results were obtained by Haines (38*a*). Tables II and III show that no phage and very little gelatinase is produced in the presence of excess phosphate or oxalate ions which remove the calcium ions. After several subcultures in such solutions the culture no longer produces phage when returned to the control solution without phosphate or oxalate but the gelatinase is formed again under these conditions. Excess calcium also prevents phage formation but does not decrease gelatinase formation markedly.

Increase in Phage without Cell Growth.—Gratia and others early pointed out that increase in phage occurred only when the host cells were increasing (39). Krueger and Northrop (23) found that this relation held quite accurately but that the ratio of the increase in phage to that of bacteria varied somewhat under different conditions indicating that it might be possible

TABLE II

Effect of Phosphate or Oxalate on Phage or Gelatinase Production

Sodium oxalate or sodium phosphate added to 100 ml. peptone. pH adjusted to 7.6. Autoclaved. Inoculated with 1×10^8 bacteria per ml. and 100 plaques per ml. Shaken at 35°C.

Time	0			M/100 sodium oxalate			M/50 sodium phosphate		
	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.	10^8 bacteria per ml.	Plaques per ml.	Gel. U. per ml.
hrs.									
0	1.0	10^2	0.004	1.0	10^2	0.004	1.0	10^2	0.004
3	2.3	10^5	0.006	2.3	20	0.002	2.4	0	0.005
5	4.5	2.7×10^3	0.40	3.5	0	0.05	2.7	0	0.02

TABLE III

Effect of Addition of Calcium Chloride

100 ml. yeast extract plus calcium chloride, pH adjusted to 7.6. Boiled and filtered. 50 ml. filtrate plus 1×10^8 bacteria per ml. plus 2.6×10^2 plaques per ml. Shaken 35°C.

Final concentration calcium chloride added	0			0.012			0.025			0.05			0.10		
Time	10^8 b. per ml.	10^2 pl. per ml	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml.	Gel. U. per ml.	10^2 b. per ml.	10^8 pl. per ml.	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml	Gel. U. per ml.	10^8 b. per ml.	10^2 pl. per ml	Gel. U. per ml.
hrs.															
0	1.0	2.5	0.004	1.0	2.6	0.003	1.0	3.0	0.004	1.0	2.7	0.004	1.0	2.6	0.004
3	2.5	10,000		2.4	10,000		1.6	10,000		1.8	8,000		1.7	2,000	
24			0.07			2.0						1.0			0.7

to separate phage increase from cell growth. Such a result is to be expected from the analogy between phage and enzyme production since microorganisms may produce enzymes (17–18, 29) under certain conditions without cell division. Pepsin and trypsin may be formed from their precursors *in vitro* (28). Thus, if any real relation exists between phage produc-

tion and enzyme formation it should be possible to obtain increase in phage without cell growth and probably even in cell free extracts. Krueger and his co-workers (30–31) was subsequently able to separate the two variables and obtain increase in phage without any increase in cells and also in cell free extracts (50). The increase in phage without cell growth can only be obtained when "young" cells are used. This result is in striking agreement with the results of Hegarty (48) who found that only young cells could produce new enzymes in the presence of new substrates. Krueger's experiments (30–31) were done with *Staphylococcus* phage and have been repeated and confirmed in this laboratory. A large number of unsuccessful attempts to repeat these results with *B. megatherium* were made under a variety of conditions. Eventually, however, it was found that such an increase could be obtained by adjusting rapidly growing cultures to a very narrow range of pH near 5.5. In this narrow range there is rapid increase in phage with a slight decrease in cells. In very slightly more acid solution both cells and phage decrease while in slightly more alkaline solution both cells and phage increase.

The results of such an experiment are shown in Fig. 9. The number of bacteria decreases slightly at pH 5.43, 5.52, and 5.58. The plaque count decreases at 5.43 but increases more than 50 times in an hour at pH 5.52 and 5.58. At 5.64 there is some increase in cells. Control experiments without phage give the same figure for the changes in bacteria concentration as in the cultures which contained phage.

Phage Production during Growth or Lysis.—d'Herelle (39, 40) found that the plaque count in growing cultures increased in steps or "bursts" and this observation has recently been confirmed by Ellis and Delbrück (41). d'Herelle considered that the phage particle multiplied in the bacteria cells and that the cell then burst liberating a large number of phage particles. These particles attached themselves to other bacteria and the process was then repeated.

Burnet (42) tested this hypothesis by determining the increase in plaque count in small samples containing originally one or two plaques but the results are not very convincing owing to the small number of counts made. A more serious difficulty lies in the fact that the percentage of samples showing positive results increased from 40 to 100. This result shows either that the method used will not detect one particle or else that the phage increases, not from 1 to 40 particles but from 0 to 40. If the method cannot detect a single particle the results indicate that a certain minimum concentration of phage is needed to give positive results and the sudden increase in plaque count occurs when this minimum is overstepped.

In any case there are no data to show that the sudden increase occurs during lysis as the number of bacteria was not determined.

Doerr and Grüninger (53), Bronfenbrenner (39), and Krueger and Northrop (23) found that the principal increase in phage occurs during growth of the bacteria and very little after lysis starts. Similar results were obtained by Clifton and Morrow (52). Krueger and Northrop considered that lysis occurred when the phage per bacteria ratio reached a certain critical value. In confirmation of this idea they found that the

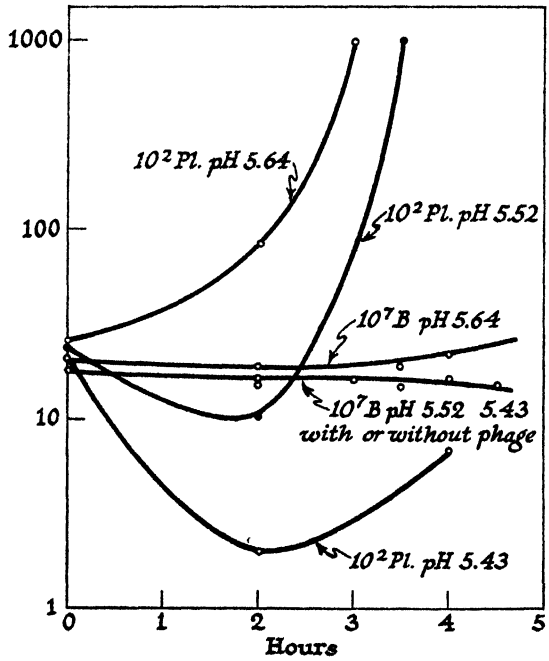

FIG. 9. Increase in plaque count without cell growth at pH 5.52. 100 ml. yeast extract inoculated with 10×10^7 bacteria per ml. from 18 hour 30°C. Blake bottle. Suspension shaken at 35°C. for 2 hours. 20 ml. aliquots titrated to pH 5.43, 5.52, and 5.64. 25×10^2 plaques per ml. added and suspension shaken at 35°C.

addition of very concentrated phage to growing cultures caused immediate lysis and that under these conditions no increase in phage occurred. This experiment has been repeated by the writer with the result shown in Fig. 10. The results show that lysis starts immediately after the addition of the concentrated phage. The curve is a typical probability integral and when plotted in the differential form gives the usual probability curve. This is the result usually obtained when a culture of bacteria is killed. If lysis is caused by the increase of the phage particle inside the cell there should be

a lag period of 20–40 minutes before any lysis occurs and the phage concentration should increase. Actually there was a slight decrease in phage instead of an increase.

The method used by Krueger and Northrop (23) determined total phage and not infective units as does the plaque count method. No sudden in-

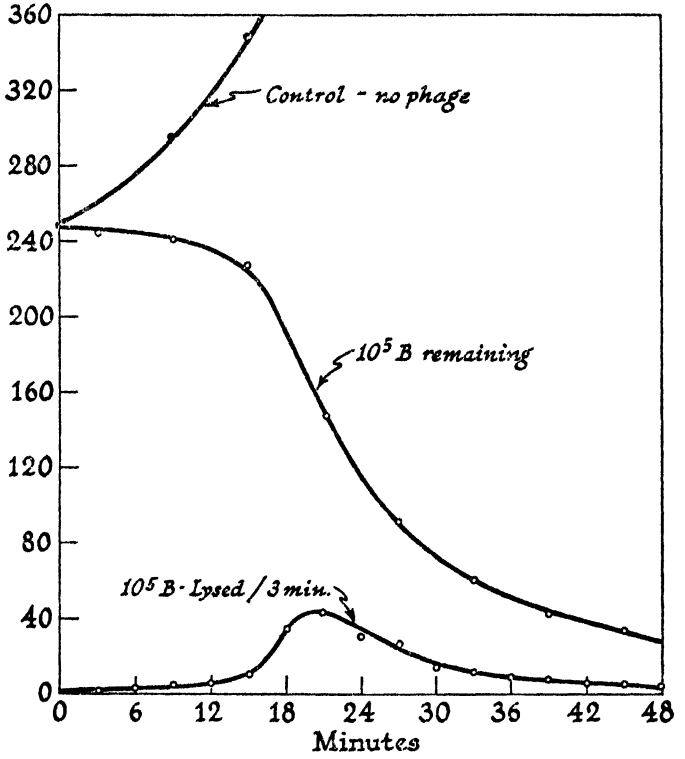

FIG. 10. Lysis of suspension of *Staphylococcus muscae* upon addition of concentrated phage solution. 50 cc. yeast extract containing 100 × 10^5 bacteria per ml. shaken at 35°C. for 2 hours. 1 cc. purified concentrated bacteriophage (6) solution containing 10^{12} plaques per ml. added. Decrease in bacteria concentration determined by turbidity method. Initial plaque count per ml. suspension 2 × 10^{10}, final plaque count 1 × 10^{10}.

crease at lysis would be predicted by d'Herelle's hypothesis when the total quantity is determined since it is assumed that there is merely a redistribution of phage at that time. Also no lysis could be detected during the upward part of the growth curve when most of the phage is formed. Ellis and Delbrück account for this by assuming that the percentage of bacteria lysed at this time is so small as to escape detection.

With the resistant strain of *B. megatherium* used in the first two experiments reported no lysis can be observed at any time yet the plaque count reaches a height of more than 10^9 per ml. This figure is 3–4 times that of the total cell concentration. If the phage particles have been liberated by undetected lysis of cells it must be supposed that each cell liberates large numbers of phage particles.

It is difficult, from this point of view, to account for the increase in both bacteria and phage from one infected organism. If the phage increases only when the organism undergoes lysis then one infected organism could give rise to either more phage or more cells but not both. If the cell lyses more phage will be liberated but no cells will be present. If the cell does not lyse there will be more cells but no increase in phage. However, single cells of this lysogenic strain give rise to colonies as well as to more phage (55).

If the percentage of organisms lysed during the growth period is very small the percentage of phage formed during this period must also be very small if it is assumed that each lysed cell liberates a constant amount of phage. According to this idea the principal increase in plaque count must occur after visible lysis starts and the increase in plaque count must be roughly proportional to the decrease in the number of cells. If, on the other hand, the phage is produced on or in the growing cells then the principal increase in phage will occur during growth instead of during lysis. This relation is best shown by plotting phage concentration *versus* bacteria concentration. If phage is liberated during lysis such a curve should rise only a few per cent as bacteria increase and the principal increase in phage should appear as bacteria decrease. This will give a curve of type L in Fig. 11. If, on the other hand, phage is formed and liberated continuously by the growing bacteria then the phage count should increase rapidly as bacteria increase and slowly or not at all as lysis starts and bacteria decrease. This will give a curve of the type G in Fig. 11. The results of ten experiments are plotted in Fig 11. All the curves but two (27 and 93) are of the G form. In the two L curves the amount of phage formed per bacteria lysed is small and the form of the curve is determined by only one point. These curves are similar to those found by Krueger and Northrop with *Staphylococcus* phage.

The decrease in phage during lysis cannot be ascribed to an inhibiting substance since filtrates from growing cultures or from cultures which have been dissolved by lysozyme do not cause a decrease in plaque count. If anything there is an increase in the presence of such filtrates.

In the case of *megatherium* and of *Staphylococcus*, therefore, it cannot be assumed that phage is liberated during lysis unless some secondary hy-

pothesis is added. In the case of some strains of *B. coli*, however, there is an increase in phage during lysis and the curves are of the L type (personal communication from Dr. Ellis).

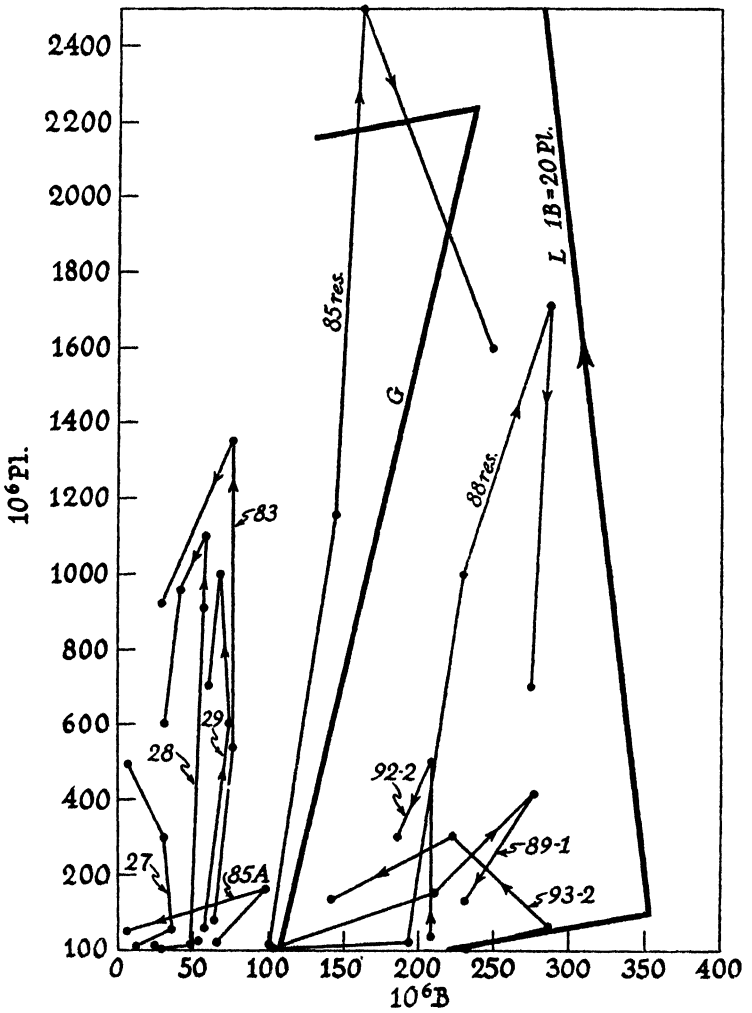

FIG. 11. Changes in bacteria and phage concentration during growth and lysis of various cultures. The number of bacteria per ml. has been plotted against the plaque count per ml. If the plaque count increases during growth of the organism the curve must rise sharply with a positive slope and a sharp break at the time the bacteria concentration decreases. The general shape will be that of curve G. If the plaque count increases during lysis the curve will have a small positive slope during growth of the cells and the principal increase in plaque count must occur when the cell concentration decreases. Most of the curve, therefore, must have a negative slope as in curve L.

Several curves have been obtained which show the step-like form described by d'Herelle and Ellis and Delbrück but there have always been slight increases in cell concentration corresponding to the sudden increase in phage concentration (Figs. 5 and 6). Since changes in bacterial growth rate are greatly exaggerated in phage increase rates a very small change in bacteria may make a very large change in phage.

These step-like curves are found only in cultures which contain resistant forms and not in cultures like *Staphylococcus* where lysis is complete and no resistant form appears. It is suggested that the step-like curves are due to the presence of two or more strains of bacteria or two or more phages of different rates of increase.

It was noted by Burnet that some phage solutions gave a longer lag period than others and this observation has been confirmed in the course of this work. Obviously the presence of these two types of phage will give step-like curves.

Experimental Methods

Bacterial Concentration.—The light absorption of a series of standard suspensions of known bacterial content was compared with that of a solution of M/25 copper sulfate in M/10 sulfuric acid in a Klett photoelectric colorimeter. The ratios of the colorimeter readings were then plotted against the cell content of the standard suspension. The bacterial concentration of the unknown sample was determined by comparing the unknown with the copper sulfate and reading off the equivalent bacterial concentration from the copper sulfate-standard suspension curve (Fig. 12).

Preparation of Standard Suspension.—A rapidly growing suspension in 2 per cent peptone was analyzed for total cell count by direct microscopic count, number of colonies by plating on agar, and total dry weight by washing and drying to constant weight at 100°C. The suspension contained 1×10^8 cells per ml., usually in clusters of 4–8 cells; 0.2×10^8 colonies and 0.2 mg. dry weight.

The dry weight content of different suspensions may be determined within a few per cent by this method but the cell count per milligram dry weight varies quite widely owing to the difference in the size of the bacteria. The results have been expressed in terms of number of cells in a standard suspension having the same light absorption in order to show the approximate relation between the actual number of cells and the number of phage plaques. They are accurate to a few per cent for the total quantity of bacteria expressed in milligrams but there may be a considerable error in the number of cells. The method does not distinguish between living and dead cells.

Example of a Determination.—1 ml. unknown suspension added to 19 ml. one per cent formalin and read against M/25 copper sulfate in M/10 sulfuric acid in Klett photoelectric colorimeter. Copper sulfate reading = 32, suspension = 20, ratio $\frac{\text{copper sulfate}}{\text{suspension}}$ = 1.6. From Fig. 1 this is equivalent to 0.02 mg. dry weight per ml., or 1×10^7 bacteria per ml. This is the concentration of bacteria per milliliter of the dilution in formalin. This is 1/20 the concentration of the original suspension so that the original

suspension contained 20 × 0.02 = 0.4 mg. ≈ 20 × 10^7 bacteria per ml. If the culture media is colored or if low dilutions are used it is necessary to correct for the color by making a determination on the culture media alone.

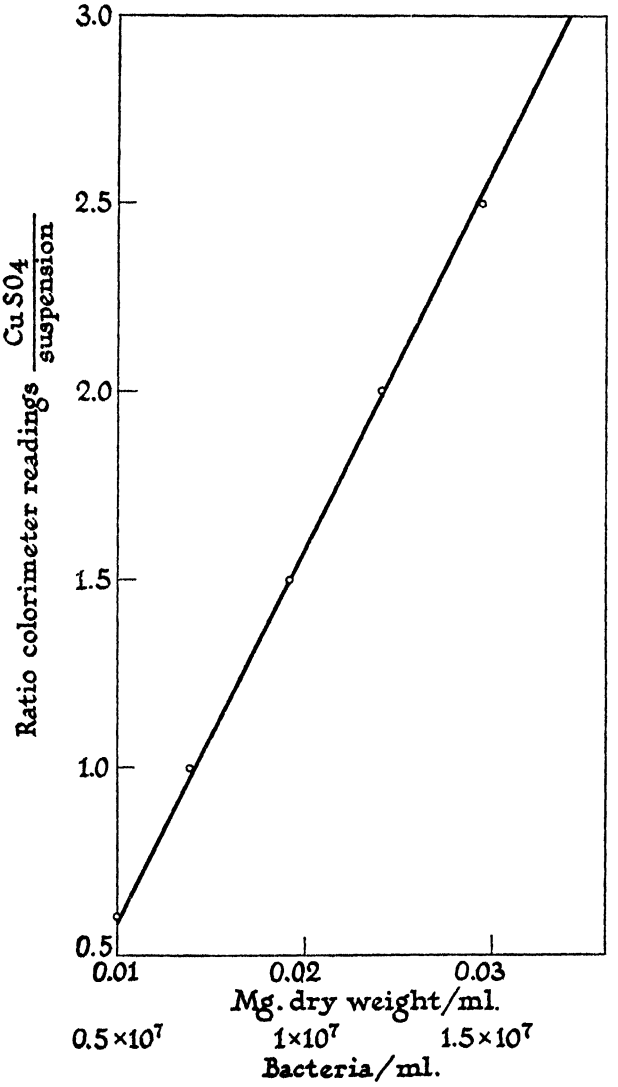

FIG. 12. Comparison of the light absorption of M/10 copper sulfate and M/25 sulfuric acid against suspension of washed bacteria in 10 per cent formalin. Ratio of colorimeter readings is plotted against the milligrams dry weight per ml. 0.01 mg. dry weight per ml. is equivalent by direct microscopic count to about 0.5 × 10^7 bacteria per ml. The results on a dry weight basis are accurately reproducible. The cell count in different suspensions may vary considerably owing to difference in the size of the cells.

Phage Determination.—The time of lysis method described by Krueger (43) cannot be used with *B. megatherium* since lysis is too slow and incomplete to allow accurate determination of the time required for lysis. The plaque count method was therefore used following the elegant technique described by Gratia (32). This method gives very accurate and reproducible plaque counts. The most accurate counts are obtained with 200–500 plaques per plate. Under these conditions the probable error of the average of 5 plates is less than 10 per cent. The results by this method are complicated by the fact that what is determined is the number of infected cells rather than the concentration of phage itself. Thus a cell which is infected will give rise to one plaque no matter how much or little phage it contains. Since the *megatherium* cell grows in clumps a further complication arises because a clump containing one or several infected cells will give one plaque in either case. The results also vary somewhat depending upon the agar concentration, as Bronfenbrenner and Korb (44) found.

Indicator Culture.—The sensitive strain of *megatherium* 36 was used in making plaque counts. The culture was kept on broth agar slants, incubated 24 hours at 30°C., and stored at 6°C. Transplants were made once a week. If transferred every day the culture may become resistant. The growth was washed from the slant with 5 ml. sterile yeast extract and broth agar Blake bottles were inoculated with 1 ml. of the suspension and incubated at 30°C. for 18 hours. The growth was washed off in 10 ml. of yeast extract and diluted to 0.1 mg. bacteria per ml. (5×10^7 bacteria) and shaken 1 hour at 35°C. in Florence flasks. It was then allowed to stand at 20°C. and 3.5 ml. used to prepare the plate as described by Gratia. Control experiments showed that the count varies with the condition of the culture. Thus, if the suspension is used immediately after washing off the Blake bottle the count is about one-half that which is obtained after 1 hour shaking. After this time the culture gives constant counts for 15–20 hours and then gives lower results again.

Culture Media. 1. Peptone.—2 per cent Fairchild's peptone plus 1 per cent sodium chloride pH 7.6, no phosphate. No phage is formed in some preparations of this peptone although good growth is obtained. Addition of 0.01–0.02 M calcium chloride may cause phage production but the results are irregular.

2. Yeast Extract Media.—1 ml. glacial acetic acid added to 1 liter distilled water and boiled. 40 gm. Fleischmann's dried yeast added and suspension boiled ½ hour. Filtered. Filtrate titrated to pH 7.8 and boiled 15 minutes. Cooled to 10°C. for 24 hours. Filtered and autoclaved. This media gives luxuriant growth of *Staphylococcus*, typhoid, *coli*, *megatherium*, and many other bacteria.

Cultural Conditions.—Krueger and Northrop (23) found that much more regular results were obtained with *Staphylococcus* culture and phage if the culture were shaken. Gratia (32) has noted the same result with *megatherium* and this result has been confirmed in the present work. Merrill and Clark (38) found greater production of gelatinase in aerated cultures. The culture was grown in 250 ml. Florence flasks containing 100 ml. or less culture media. They were rocked in a water bath at the desired temperature so as to cause violent agitation. Under these conditions the bacteria concentration doubles in less than 1 hour and a maximum concentration of $4–6 \times 10^8$ bacteria per ml. equivalent to 1 mg. per ml. dry weight is reached. If the culture is not shaken growth is very much slower and never reaches such high values.

Gelatinase Determination. Gelatin Solution.—25 gm. air dry isoelectric gelatin added to 500 ml. $\begin{cases} \text{0.015 M ammonium acetate} \\ \text{0.01 M sodium hydroxide} \end{cases}$. Heat to 55°C., adjust to pH 7.6. Filter and store at 0°C. For use warm to 80°C. for 5 minutes, pipette 5 ml. in test tube, cool to 35°C. for 10 minutes or longer. 1 ml. culture added to 5 ml. gelatin, solution poured into Ostwald viscosimeter and viscosity determined at intervals. The time of outflow is plotted against the elapsed time and the per cent change in the relative viscosity per minute interpolated from the curve. One gelatin unit (gel. u.) is defined as a change in the specific viscosity of the gelatin solution at the rate of 1 per cent per minute (51).

Most of the experimental work reported in this paper was done by Miss Elizabeth Shears and Mr. J. F. Gettemans.

SUMMARY

1. The increase in bacteria, phage concentration, and gelatinase concentration in cultures of *B. megatherium* has been determined.

2. With lysogenic cultures the phage concentration, gelatinase concentration, and bacteria concentration increase logarithmically at first. The phage and gelatinase concentration then decrease while the bacteria concentration increases to a maximum.

3. The results are the same with sensitive cultures if the ratio of phage to bacteria is small. If the ratio of phage to bacteria is large phage, gelatinase, and bacteria concentration all increase at first and then decrease. The maximum rate of increase coincides approximately with the maximum rate of oxygen consumption of the culture.

4. 60-90 per cent of the phage is free from the cells.

5. The amount of phage produced is determined by the combined phage and not by the total phage.

6. Phage is produced during growth of the cells and not during lysis.

7. In a very narrow range of pH near 5.55 no increase in bacteria occurs but large increases in phage may be obtained.

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CHEMICAL PACEMAKERS

PART I. CATALYTIC BRAIN IRON. PART II. ACTIVATION ENERGIES OF CHEMICAL PACEMAKERS

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(Received for publication, May 31, 1939)

PART I

Catalytic Brain Iron

Merritt, Moore, and Solomon (1933) have reviewed literature dealing with the occurrence of precipitated spicules of iron in the brains of patients who have died with dementia paralytica (tertiary brain syphilis). The following quotations are from their paper, which should be consulted for citations.

"Iron pigment is found in all normal brains in a fairly characteristic distribution. This iron is spoken of as endogenous iron. . . . When normal brain is treated with the Turnbull-blue method this iron stains in a rather diffuse manner and is either free in the tissues or within the oligodendroglia. Wuth has studied the distribution of this iron chemically and has shown that the amount found in the different regions of the normal brain parallels the results obtained by the histopathologic method (Turnbull-blue reaction). . . .

'In dementia paralytica the iron is found in a very characteristic [and different] manner as follows: when brain tissue is stained with the Turnbull-blue method small spicules of iron pigment are found in the cells of the blood vessel walls and in the body or processes of the microglia cells. This iron is not localized in any one portion of the brain but is distributed diffusely through the cortex, basal ganglia and other regions . . . and grossly parallels the intensity of the specific inflammatory process. . . .

'As to the specificity of the reaction it must be stated that Spatz and Metz have described similar iron deposits only in the brain of patients who died of African sleeping sickness, which, it should be remembered, is a trypanosomal disease and is often indistinguishable histopathologically from dementia paralytica. . . .

'In dementia paralytica iron deposits are found chiefly in the cortex and in the corpus striatum. Jahnel . . . states, 'The iron reaction is positive without exception in typical dementia paralytica.' . . . In slowly developing or in 'stationary' cases, the iron reaction is commonly very weak and at times one may miss it. . . .

'The source of the pigment is still not clear. Its origin from blood cells is not acceptable because usually one does not find hemorrhage, diapedesis or red cells undergoing phagocytosis in cases of dementia paralytica. . . .

'This question of iron pigment in dementia paralytica is of paramount importance in relation to the pathologic process of dementia paralytica. . . .' "

We wish to indicate that the above findings are consistent with the view that the precipitated iron spicules found in brains of general paretics come from a transformation of *part* of the catalytic iron of the brain to a precipitable inactive form as a result of the infection. This iron is normally present in hematin derivatives such as cytochrome. The brain is especially well supplied with cytochrome-cytochrome oxidase.

Menkin (1935), Menkin and Talmadge (1935), and Lorenz and Menkin (1936) have discussed the development of iron pigment, which does not come from hemoglobin break-down, accompanying a variety of pathological conditions in tissues other than the brain.

If our view is correct, the total iron in the brains of general paretics and controls should be the same. If the iron granules of paretic brains come from the blood, the total brain iron of this group should be larger. Freeman (1930) showed that schizophrenic patients have a significantly smaller amount of total brain iron than have other patients. In his published data the analyses of eight cases of general paresis are, fortunately, included. We have eliminated the schizophrenic group (having low iron) and one case of pernicious anemia and have averaged the remaining twenty-four non-general paresis cases as controls. This control group gives a mean of 47.6 mg. Fe per 100 gm. dried cortex, with a standard deviation of ± 0.554. The eight general paretics give 48.3 mg. Fe per 100 gm. dried cortex with standard deviation of ± 0.891. The difference of the means is 0.9. The σ difference (square root of the sum of the two standard deviations squared) is 1.05. Hence the mean difference of 0.9 is entirely without significance and the total iron content of the brains of the paretic group and the controls is not different.

On the physiological side, studies of temperature characteristic of alpha brain wave frequencies in normal man and in general paretics, whose temperatures were raised by diathermy, furnish direct evidence for the view that catalytic iron is inactivated in advanced stages of paresis. The Arrhenius equation, $v = ze^{-\mu/RT}$, describes the speed of a variety of chemical processes as a function of temperature, where v is chemical velocity, e is the base of natural logarithms, T is the absolute temperature, z is a constant, R is the gas constant, and μ is the critical thermal increment or energy of activation; *i.e.*, the amount of energy per mol above the average energy in the system necessary to render molecules reactive.[1] The values of μ recorded in the

[1] In physical chemical literature E is used in place of μ for the energy of activation. To avoid the implication that the μ of biological processes is necessarily identical with the energy of activation as used by the physical chemists, Crozier has preferred to speak of μ as the "temperature characteristic" rather than the energy of activation. The

literature for cell respiration *in vitro* for seventy-four different sets of experimental data fall in some six modes (two of which are very distinctive) when their distributions are plotted (Hoagland, 1936*a*). The principal modes are twenty values (27 per cent) at 16,500 ± 500 calories; thirteen (18 per cent) at 11,500 ± 500 calories; and four (5 per cent) at 8500 ± 500 calories. Earlier Crozier (1925-26) made a distribution plot of some 360 μ values for diverse physiological frequencies (heart rates, respiratory movements, *etc.* of poikilothermal animals) which also conform to the Arrhenius equation. The modes are of the same order as are found for cell respiration. For these data ninety-six (twenty-seven per cent) occur at 16,500 ± 1000 calories; eighty-one (12 per cent) at 11,500 ± 1000 calories; and thirty-two (nine per cent) at 8000 ± 500 calories. Thus for different physiological frequencies 58 per cent of 360 μ values fall within the 8, 11, 16 thousand calorie groups and 24 per cent fall within other minor but definite modes. For direct respiration determinations of CO_2 production and O_2 consumption 50 per cent of seventy-four determinations fall within these three major groups and 34 per cent in minor μ groupings. Chi square tests have demonstrated the statistical reality of the modes (Hoagland, 1936*b*).

If frequency (with the dimension of reciprocal time or rate) be proportional to some underlying chemical velocity, we should expect to get the same μ value for frequency as for a direct determination of the velocity. This follows from the nature of the equation.

If $v = ze^{-\mu/RT}$, taking logarithms we obtain, $\log v = c - \mu/2.3\ RT$, and, if the data fit the equation, a plot of $\log v$ against $1/T$ should give a straight line, with intercept c and negative slope $\mu/2.3\ R$. If the frequency is proportional to a chemical velocity, $f = kv = kze^{-\mu/RT} = ae^{-\mu/RT}$, and again taking logarithms we get, $\log f = c' - \mu/2.3\ RT$. Thus a plot of $\log f$ *vs.* $1/T$ would give the *same* μ *value* as if we plotted $\log v$ *vs.* $1/T$. In a log frequency plot the intercept on the ordinate would be at c' instead of at c, *i.e.* the two plots would give parallel lines of the same negative slope equal to $\mu/2.3\ R$, or $\mu/4.6$, since R is 1.99 or 2 calories per mol per degree.

To account for the often recurring μ values in different protoplasmic systems, Crozier (1924-25) originally suggested that the modal values may correspond to energies of activation of specific catalyzed links in sequential steps in chemical chains constituting cellular dynamics. The wide recurrence of certain respiratory enzymes in highly diverse tissue systems is in

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present writers refer to μ as activation energies for reasons that will appear in the following pages although it should be borne in mind that the concept of energy of activation of an enzyme system may not be identical with that as used by physical chemists.

conformity with this view. Catalysts promote reactions by so orienting molecules that particular bonds can be broken in a way not possible except by a marked increase in kinetic energy produced at elevated temperatures, and on theoretical grounds there is reason to expect the catalyst to determine the μ for certain types of reactions (Hinshelwood, 1929). In a chain of catalyzed reactions we should expect the slow link to act as master reaction or chemical pacemaker, thus determining the velocity as a whole. If in different cell systems the slowest link is now one and now another of a few enzyme systems common to many different tissues, a multimodal distribution of μ values, such as has been obtained, would be anticipated. Moreover experimental conditions altering the ratio of velocity constants of the respiratory steps might be found to shift the μ value from one of the prominent characteristic group values to another. Such experimental shifts of μ from one of the 8, 11, or 16 thousand groups to another have been described (Hoagland, 1936 *b* for citations).

Most physiological rates conform to the Arrhenius equation in that they give rectilinear relations when log rate is plotted *vs.* $1/T$. For systems involving two or more *concurrent* chains of processes with different μ values, each process of which contributes appreciably to the measured rate, we should not expect a rectilinear relation. In such cases the Arrhenius plot gives a curve which is concave upwards (Crozier, 1924–25).[2] When such concurrent processes are analyzed separately they are found to give rectilinear relations (Hinshelwood, 1929[3] for examples). Runnström (1930) has shown that the respiration of fertilized *Arbacia* eggs involves two concurrent processes. Korr (1937) has found the type of concave curve to be expected under these conditions. From Korr's data Crozier (unpublished) has calculated the effects of temperature on each of the two independent concurrent chains of processes separately. These separated processes give excellent rectilinear relations according to the Arrhenius equation. The μ value for the process involving the cyanide-sensitive system is approximately 17,000 calories, that for the other concurrent chain is approximately 8,000 calories.

Hoagland (1936 *a*) reported that normals and early general paretics give, for alpha brain wave frequencies $\mu = 8000 \pm 200$ calories; more advanced paretics give $\mu = 11{,}000 \pm 300$ calories, and very advanced paretics give $16{,}000 \pm 300$ calories. The results suggest that these μ values are energies of activation of particular chemical pacemaker links in respiratory reaction

[2] Crozier, W. J., 1924–1925, *J. Gen. Physiol.*, **7**, 192.

[3] Hinshelwood, C. M., 1929, The kinetics of chemical change in gaseous systems, Oxford, University Press, 45.

chains in the cells producing the rhythms. Evidently the advancing spirochete infection shifts the chemical pacemaker from one reaction to another.

The oxidation of ferrous to ferric iron *in vitro* requires an energy of activation (μ) of 16,200 $\pm$ calories (Noyes and Wason; Hood *et al.*, cited by Crozier, 1924–25). In many cell respiratory processes it has been demonstrated by direct chemical methods that an iron catalyzed process is often the limiting factor (Warburg, 1935) and it is known that the catalytic iron undergoes oxidation and reduction. This is, incidentally, consistent with the predominance of occurrence of approximately 16,000 calories among physiological μ values.

In view of these findings a series of experiments were carried out by Hoagland and his collaborators to test further metabolic factors underlying central nervous rhythms and the following results were obtained:

Lowering blood sugar by insulin below a critical level, and thus brain sugar, causes the alpha frequencies in man and dogs to fall. The frequencies recover when sugar is injected (Hoagland *et al.*, 1937; 1939). Brain O_2 consumption follows the curve of sugar after insulin, and the alpha frequency curve, within certain limits, parallels both the other curves (Himwich *et al.*, 1939). Dinitrophenol and thyroxin, well known metabolic stimulants, cause the frequencies to increase (Rubin, Cohen, and Hoagland, 1937; Hoagland, Rubin, and Cameron, 1939). Pentobarbital sodium, which inhibits brain respiration *in vitro*, slows the cortical frequencies (Hoagland *et al.*, 1939).

It should be borne in mind that these findings apply only to frequencies (*i.e.*, rates) and not to the amplitude factor or to the total energy of the brain waves, as may be determined by the type of analyzer described by Grass and Gibbs (1938). Introduction of variables other than that of rate in these particular considerations would not be pertinent for us. The above experiments have been carried out on men or animals showing clearly countable alpha rhythms. The probable errors of frequencies in 50 seconds of such rhythms in man are less than 1 per cent (Hoagland, 1936*a*).

These findings are of course *not* to be interpreted as meaning that the *only* modifiers of brain wave frequencies are necessarily changes in cell respiration. Afferent stimulation, which modifies frequencies, may do so by locally changing cell respiration or by modifying the electrical constants (impedance) of the cells or their connections through permeability changes. Absolute differences in frequencies of different cell groups also are not to be regarded as due to corresponding absolute differences in rate of O_2 consump-

tion, but rather to probable differences in structure of the cell walls or connecting fibers. These considerations in no way militate against the view that certain *relative* frequency changes under standardized conditions are due to changes in rates of cell metabolism. The waves may be regarded as arising in the cells independently of afferent connections, or, less likely, as due to closed or "reverberating" circuits set off initially by afferent stimuli. In the latter case oxidative recovery from the refractory state of the circuit may be pictured as important in determining the frequency.

It is probable, from what is known of other cells, that cortical cells produce potential gradients as a by-product of their respiratory metabolism. These may be of the nature of diffusion potentials across the cell membranes which possess definite electrical impedance and which discharge when the potentials reach a critical value. In such a system the discharge frequency depends on the speed with which the metabolic factor can load the capacities of the cell walls to their critical discharge potentials. The absolute frequency would thus depend on the rate of cellular respiration *and* on the electrical impedance of the cell walls. If this last be *statistically* constant for a particular group of cells under the conditions of the experiments the *frequency* should parallel, and be a measure of, cortical respiration. The nature of electrical synchronization between the cell units is a matter with which we are not here concerned.

If the general view outlined above is correct it should be possible to test it by obtaining a simple two-component oxidative enzyme system acting sequentially, containing one of the dehydrogenases common to brain, and also cytochrome-cytochrome oxidase which is plentifully present in brain. By selectively poisoning one or the other of the components and thus making more or less of each component available, it should be possible to shift the μ for O_2 uptake from one of two of the principal modal values to another characteristic of the slow step and independent of the absolute speed of O_2 consumption. When the cytochrome system is made the slow step by reducing *some* but not all of its activity with a poison such as cyanide, which does not affect the dehydrogenase link, the theory requires that the μ value be approximately 16,000 calories.

PART II

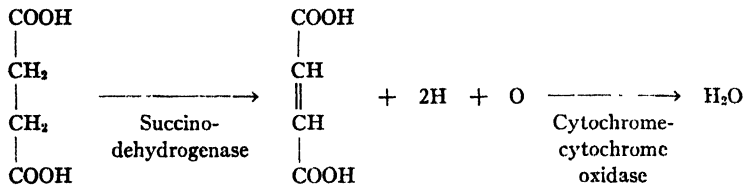
Activation Energies of Chemical Pacemakers

Sizer (1937) and Gould and Sizer (1938) have demonstrated that μ values of biological processes may be indicative of specific enzymes. Sizer has shown that the hydrolysis of sucrose and of raffinose by yeast invertase

gives a μ value of about 12,000 calories, which is independent of pH changes ranging between 3.2 and 7.9 units and is not altered by the presence of electrolytes. Gould and Sizer have shown that the decoloration of methylene blue by the action of *Escherichia coli* in the presence of ten widely differing substrates yields a μ value of 19,400 calories for the dehydrogenations and is thus independent of the gross nature of the substrate (see Moelwyn-Hughes, 1933).

If some process catalyzed by an enzyme system containing two or more components acting in sequence were to be studied *in vitro*, it should be possible to obtain different μ values for the process by partially poisoning different components of the system, thus making now one and now another step the slow one. Furthermore, if one of these components be an iron-containing enzyme, one of the two μ values may be expected to be 16,000 $\pm$ calories (see above). The objection might be raised that the enzyme-catalyzed processes *in vivo* need not necessarily correspond to such processes *in vitro*. This objection has been met by Sizer (1938) who has shown that the invertase inversion of sucrose by living yeast cells and by yeast cells killed in toluene yields identical μ values.

Such a desired enzyme system may be extracted from the beef heart by methods described by Stotz and Hastings (1937). This system has been shown by these workers to oxidize succinate to fumarate and to involve at least two components, a dehydrogenase and cytochrome-cytochrome oxidase. The reaction for our purposes may conveniently be written as follows:



In this reaction succinic acid is oxidized to fumaric acid, and oxygen activated by the oxidase system is consumed. The oxygen combines with hydrogen from the succinate activated by its dehydrogenase. The system is of special interest since it is almost universally present in tissues and plays a prominent rôle in carbohydrate metabolism (Szent-Györgyi, 1937). Both succino-dehydrogenase and cytochrome-cytochrome oxidase are amply present in brain (Page, 1937), thus directly relating matters discussed in Part I with present considerations.

Stotz and Hastings have shown (1) that the enzyme as prepared by them

is free of fumarase, since the amount of fumarate formed agrees stoichiometrically with the oxygen consumed, indicating that none of the fumarate formed by the oxidation of succinate is hydrolyzed to malic acid. (2) The reaction follows first order kinetics. The value of the velocity constant does not deviate from the mean significantly for the first 85 per cent of the reaction, thus indicating that there is no destruction of the enzyme, at least for the first 85 per cent of the reaction. (3) The addition of 6.0×10^{-6} M NaCN or 2.0×10^{-5} M selenite per 0.5 ml. enzyme solution stops the oxidation of succinate completely. (4) Oxidase activity can be studied independently, since cytochrome-cytochrome oxidase can oxidize *p*-phenylenediamine without a dehydrogenase and the dehydrogenase activity can be studied independently if a hydrogen acceptor, such as methylene blue requiring no activating catalyst be substituted for oxygen and the cytochrome system. These studies show that the addition of 6.0×10^{-6} M NaCN stops the oxidase activity completely and does not diminish the dehydrogenase activity, while the addition of 2.0×10^{-5} M selenite stops the dehydrogenase activity completely and does not diminish the oxidase activity. (5) The addition of less NaCN or less selenite partially and selectively inactivates one or the other of the components.

These observations show that the oxidation of succinate to fumarate by the beef heart extract proceeds in at least two steps. Two different enzymes are required, which act sequentially. If the view is correct that in such a system the speed of the slower step determines that of the overall reaction (in this case the rate of oxygen consumption), and if μ values are associated with enzymes as discussed above, then we may expect it to follow that (1) the enzyme system as extracted from beef heart should yield a μ value characteristic of the particular step which is the slower. (2) By partially poisoning the other (faster) component of the enzyme system, a different μ value corresponding to this now slower component should be obtained. (3) This shift in μ should come abruptly with increasing concentrations of the poison. (4) The μ value should shift back to the original, if both components are poisoned such that the original slow step is restored to this position. (5) A μ value of about 16,000 should be obtained when the cytochrome-cytochrome oxidase component, which contains catalytically active iron, is poisoned sufficiently to make this step the slow step.

The investigation was undertaken to determine whether these deductions could be verified experimentally. All of the five points have been tested and the results have been found to conform entirely to the predictions.

Procedure

Preparation of Enzyme.—The method of Stotz and Hastings was employed without modification in extracting the enzyme system from beef heart.

Measurement of Oxygen Consumption.—Reactions were carried out at constant temperature in Erlenmeyer type Warburg vessels, and the oxygen consumption was measured manometrically. Each vessel contained 1.5 ml. M/15 phosphate buffer (pH 7.4), 6.0×10^{-5} M sodium succinate, NaCN, or selenite in those experiments involving the poison technique, and enough distilled water to bring the volume up to 2.5 ml. In the side arm was placed 0.5 ml. of the enzyme extract. The vessels were adapted to the temperature of the bath for at least 10 minutes before starting the reaction. It was found that for speed and accuracy of measurement, a mixture of buffer, succinate, distilled water, and poison (whenever used), could be prepared a day or two previous to the experiment, and 2.5 ml. of this mixture pipetted into each vessel.

To start the reaction it is necessary to take the vessels out of the bath and tip them to mix the enzyme with the rest of the reaction mixture. This takes some seconds, and since there may be as many as five reaction vessels in use simultaneously, the last reaction may not be started until as much as 45 seconds after the first. Errors in time measurements due to this factor were eliminated to a great extent by taking the readings in the same order in which the reactions were started. Since this takes approximately as much time as starting the reaction such errors could not have been greater than 10 seconds. Furthermore, taking the vessels out of the bath produces slight temperature changes. For these two reasons accurate measurements at the very beginning of the reaction could not be made. Readings were taken every 5 or 10 minutes.

Determinations at a significant number of different temperatures were carried out within as short a period as possible (15 hours), and the first determination usually was repeated to ascertain that the activity of the enzyme had not changed appreciably during the day. If this change was over 10 per cent, the experiment was discarded. Usually the change was much less than this. To compensate for these errors, temperatures were selected at random throughout the day.

Measurement of Velocities of Reactions.—It has been mentioned before that this reaction, if carried out under the experimental conditions specified by Stotz and Hastings follows a first order equation. It is obvious, however, that the reaction is not quite so simple, and there is no justification to expect it to follow this equation under varying conditions produced by poisoning the enzyme. It was desirable, therefore, to have some method other than the calculation of the velocity constant to determine the speed of reaction. This was done in the following manner. The oxygen consumption was plotted against time for each set of determinations. A family of curves was thus obtained. All these show an initial lag, followed by a portion of the curve which is almost linear. The slopes of the curves at some point within this range can be determined rather accurately with a tangent meter giving directly the velocity of the reactions in c. mm. O_2/min. The point of 120 c. mm. of oxygen consumption was taken in every case for this determination. This point is far enough along the reaction to be free from the effect of the "initial lag," and not too far along to make the determination time consuming.

Calculation of μ Values.—Speeds of reactions were obtained in the manner described

above and logarithms of these speeds plotted against the reciprocal of the absolute temperature. Single experiments contained no more than seven points and usually only five. It was, therefore, desirable to put several of these experiments together to obtain composite μ plots. Since absolute rates vary greatly from experiment to experiment, this had to be done by shifting the individual lines along the log V axis. The lines were fitted by "eye" and their slopes determined directly from the graphs. These slopes are negative, and, as shown in the preceding paper, are equal to $\mu/2.3R$ or,

$$\mu = 2.3R \times \text{slope} = 4.60 \times \text{slope}.$$

RESULTS

Reaction Velocities

Fig. 1 shows typical oxygen consumption curves for a series of determinations at different temperatures in a single experiment with an unpoisoned enzyme system. The velocity of each reaction was determined at the point

TABLE I

Temperature	Velocity of reaction
°C.	c.mm. O_2/min.
20	5.76
25	8.32
28	9.20
32	12.4
37	15.0
40	18.0

of 120 c. mm. oxygen consumption, indicated on the graph by the horizontal line. The results are given in Table I.

Effect of Addition of Succinate, and of Incubation of the Enzyme with Cyanide, on the Velocity of the Reaction

Before discussing the results of the temperature studies, it is necessary to mention two experiments which were undertaken in anticipation of certain objections which might be raised. Although Stotz and Hastings' work offers definite evidence that the enzyme is not destroyed in the course of reactions, it was thought desirable to test this point further by the addition of succinate to the reaction after it had progressed for some time. Furthermore, it was important to know whether incubation with NaCN for varying lengths of time would influence the rate of reaction. Since reactions at low temperatures take much longer to reach the desired point, it is obvious that progressive poisoning of the enzyme would introduce serious complications.

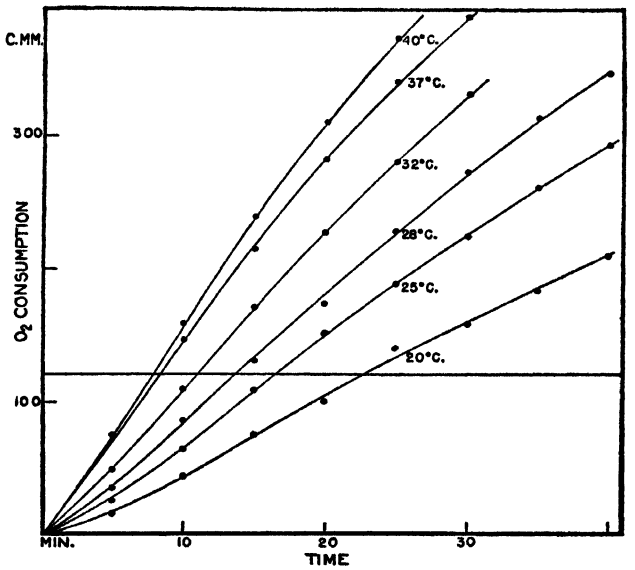


FIG. 1. Reaction curves for a single experiment with unpoisoned enzyme at different temperatures. The horizontal line indicates the points at which velocities were measured.

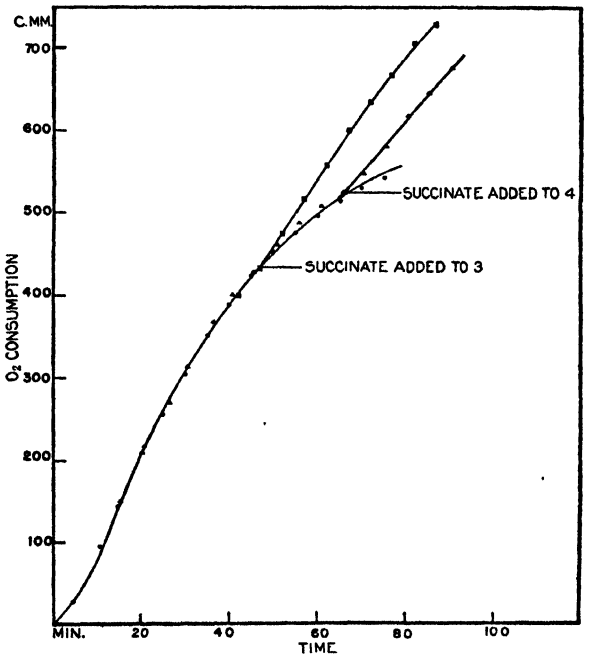


FIG. 2. Effect of addition of succinate to the reaction mixture at different intervals in the course of reaction. ● = control. ▲, ■ = 4×10^{-5} M succinate added at points indicated on the graph.

To test the effect of addition of succinate the following experiment was carried out. Four vessels were prepared, the first two (controls) containing 1.5 ml. buffer, 0.3 ml. of 0.2 M succinate, and 0.7 ml. distilled water along with 0.5 ml. enzyme in the side arm. The other two contained 1.5 ml. buffer, 0.3 ml. of 0.2 M succinate, 0.5 ml. distilled water, and 0.5 ml. enzyme along with 0.2 ml. of 0.2 M additional succinate in the side arm. The vessels were placed in the constant temperature bath at 37°C. After 15 minutes of adaptation the reactions were started in the first two vessels and the course of the reaction was followed for 75 minutes. The course of the reaction in the other two vessels (Nos. 3 and 4) was followed for 25 minutes before the addition of succinate from the side arm. It was thus possible to superimpose the reaction curves of vessels 3 and 4 before the addition of

TABLE II

January 29, 1939. Enzyme Prepared January 27. 2.4 × 10^{-7} M NaCN added. Temperature 37°C.

Time of incubation with NaCN at 37°C. before beginning of reaction—1 and 2, none; 3, 25 minutes; 4, 45 minutes; 5, 60 minutes.

Time	Oxygen consumption in c.mm.					
	1	2	Average	3	4	5
min.						
5	6.8	8.4	7	9	10	11
10	24.1	23.5	24	23	27	23
15	37.8	38.6	38	35	42	42
20	55.0	55.4	55	50	55	54
25	70.5	70.6	71	68	67	73
30	87.7	85.6	87	84	81	84

succinate on the reaction curve of the controls (Nos. 1 and 2), and so determine at what point in their courses reactions 3 and 4 were at the time of the addition of succinate. Fig. 2 shows the results. There is a definite increase in the rate of oxygen uptake with the addition of succinate, indicating that there is no appreciable change in the activity of enzyme in the course of a single reaction.

To determine the effect of NaCN incubation, five vessels were prepared. Vessels 1 and 2 contained the usual reaction mixture with NaCN and the enzyme in the side arm, while in the others the enzyme was mixed in with the reaction mixture and the succinate was placed in the side arm. The reactions in these last vessels were started after varying lengths of time. Table II shows the results of this experiment. There is no significant

difference in the rate of oxygen uptake between the average of vessels 1 and 2 in which the poisoning was practically instantaneous, and in vessels 3, 4, and 5 in which the enzyme had been incubated with NaCN for 15 to 60 minutes before the beginning of the reaction.

Results of Temperature Studies

Normal Enzyme and Enzyme Poisoned with 2.4×10^{-7} M NaCN

The results of these experiments are shown in Fig. 3. Curve I is a composite plot, made in the manner described in the procedure, of five experiments with unpoisoned enzyme extract covering a temperature range of 23°C. It is obvious that the only curve that can be drawn through the points is a straight line. The line fits not only the aggregate points, but also the individual experiments quite well. Much of the scatter of the points is probably due to an approximately 5 per cent decline in enzyme activity during the day. It should be recalled that the temperature determinations are deliberately scattered throughout the day's run. This curve yields a μ value of 11,200 calories. The fit is by eye, but a change in the slope of the line, producing a change in the μ value of 200 calories is noticeable, and would destroy the goodness of fit. Other experiments, run as controls with NaCN experiments, and yielding μ values about 11,000, but consisting of merely three points are not included in this plot.

Curve IV is a similar composite plot of five experiments with the cyanide-poisoned enzyme. In these experiments 2.4×10^{-7} M NaCN was added to each reaction mixture. Since the normal enzyme yielded a μ value of approximately 11,000, and since, according to our hypothesis, the μ value might be expected to be about 16,000 if the oxidase-activated step were the slow step, it was assumed that the dehydrogenase-catalyzed step was the slow step in the normal enzyme (a point which we shall test further). Stotz and Hastings have shown that the addition of 2.0×10^{-7} M NaCN to 0.5 ml. of enzyme inhibits the oxidase activity 64 per cent. This might be sufficient inhibition to make the oxidase-catalyzed step the slow step in the oxidation of succinate. Fig. 3, curve IV, yields a μ value of 16,100 calories, indicating that the slow step does change as expected.

Enzyme Poisoned with Varying Concentrations of NaCN.—We should expect the μ value to change abruptly with increasing concentrations of NaCN. Not until enough NaCN is added to make the oxidase-catalyzed step the slow step should the μ value shift. Fig. 3, curves II and III, show that this is actually the case. Curve II is a composite plot of four experiments with 1.2×10^{-7} M NaCN. The μ value is 11,400, which is not

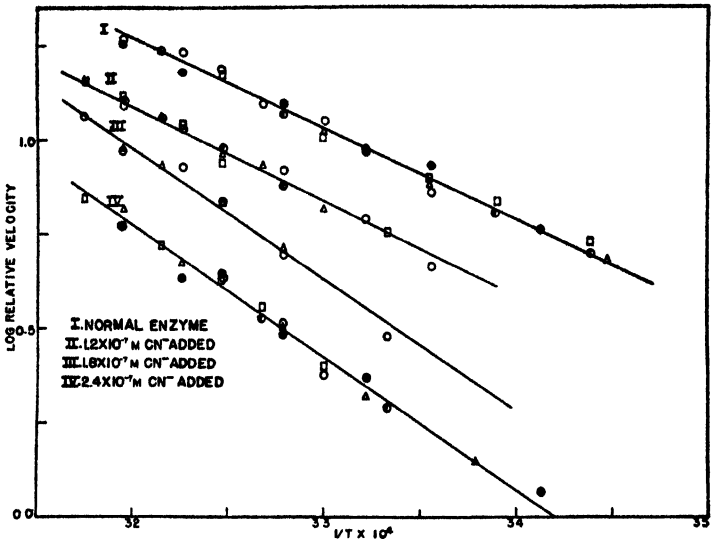


FIG. 3. Arrhenius equation plot of a group of experiments on normal and cyanide-poisoned enzyme. Individual experiments have been brought together by shifting the lines along the ordinate. Each symbol represents a different experiment.

I. μ = 11,200. II. μ = 11,400. III. μ = 16,100. IV. μ = 16,100.

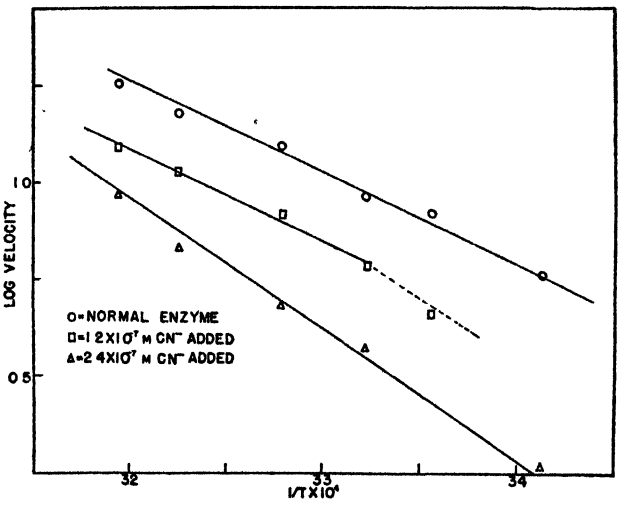


FIG. 4. Arrhenius equation plot of a single experiment on normal and cyanide-poisoned enzyme.

○—μ = 11,000.

□—μ = 11,000.

△—μ = 15,800.

significantly different from 11,200. Curve III is a similar plot of two experiments with 1.8×10^{-7} M NaCN. The μ value for this is 16,100.

Fig. 4 shows the results of a single experiment with varying concentrations of NaCN. In this experiment determinations were made simultaneously on the same enzyme preparation poisoned with different concentrations of NaCN. The lines are plotted on an absolute scale, showing changes in absolute rates as well as in μ values. The top curve shows the result of determinations with the normal enzyme. The absolute rate is 12.4 c. mm. O_2/min. at 32°C. The μ value is 11,000 calories. Addition of 1.2×10^{-7} M NaCN to the enzyme lowers the rate of the reaction (to 8.3 c. mm. O_2/min. at 32°C.), but does not change the μ value, as shown by the middle curve. This curve was purposely drawn parallel to the top curve. It fits perfectly except for the lowest point. If the concentration of NaCN is increased to 2.4×10^{-7} M, not only is the rate lowered still further (to 4.8 c. mm. O_2/min. at 32°C.), but the μ value is shifted to 15,800 calories (bottom curve).

Enzyme Poisoned with NaCN and Selenite.—If our assumption is correct, that in the normal enzyme the dehydrogenase-catalyzed step is the slow step, then the addition of selenite to normal enzyme should not change the μ value, but the addition of sufficient selenite to the enzyme system already poisoned with NaCN should yield a μ value of about 11,000. Fig. 5 gives the results of a single experiment which shows this to be the case. The top curve, the result of an experiment with the normal enzyme, yields a μ value of 11,400. Addition of 2.0×10^{-6} M of selenite lowers the absolute rate of the reaction, but does not change the μ value (second curve). On the other hand, the addition of 2.0×10^{-7} M NaCN does change the μ value to 16,200 (third curve); and the addition of 2.0×10^{-6} M selenite to the enzyme already poisoned with 2.0×10^{-7} M NaCN changes the μ value back to 11,400 (bottom curve). Changes in absolute rates are of the same order of magnitude as in the previous experiment.

Fig. 5, bottom curve, shows a definite break. These curves are plotted on an absolute scale, and it can be seen that several points on the two lower curves coincide at low temperatures. It is reasonable to expect that the concentration of selenite used does not poison the dehydrogenase component to make the dehydrogenase-catalyzed step the slow step over the entire temperature range, and that the oxidase-catalyzed step may become the slow step at lower temperatures.

In Fig. 6, curve I, we have a composite plot of three experiments in which sufficient cyanide (2.0×10^{-7} M) was used to yield the μ of 16,000 calories. Simultaneously data for curve II were obtained by using the same amount

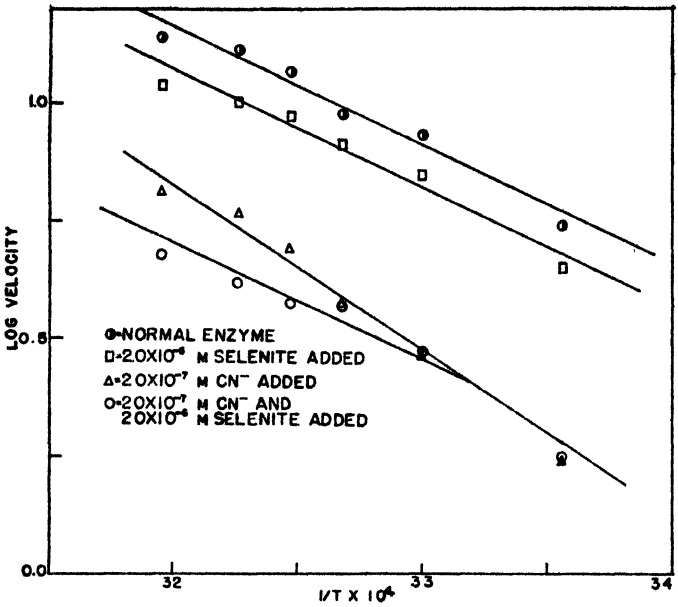


FIG. 5. Arrhenius equation plot of a single experiment on normal and poisoned enzyme.

◐—μ = 11,400.
□—μ = 11,400.
△—μ = 16,200.
○—μ = 11,400.

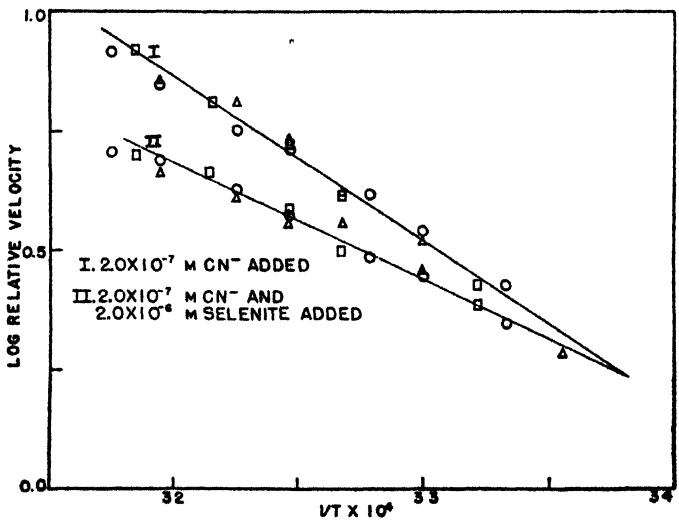


FIG. 6. Arrhenius equation plot of a group of experiments on enzyme poisoned with cyanide and with cyanide plus selenite.

I. μ = 16,000. II. μ = 11,400.

of cyanide plus enough selenite (2.0×10^{-6} M) to make the dehydrogenase-catalyzed step the slower of the two. The μ value for curve II is 11,400 calories.

DISCUSSION

It is evident that the experimental findings fully confirm the deductions made from theory. To summarize briefly the experimental results, it has been shown that: (1) the enzyme system extracted from the beef heart and containing two components yields a μ value of 11,200 $\pm$ 200; (2) this value changes to 16,000 $\pm$ 200 when the enzyme is poisoned with sufficient NaCN; (3) this change comes abruptly with increasing concentrations of NaCN; (4) the μ value shifts back to 11,200 if sufficient selenite is added to enzyme already poisoned with enough NaCN to yield a μ value of 16,000. It is safe to state that these results confirm the view that in a series of enzyme-catalyzed steps the slowest step can be the pace-determining step for the entire series.

In Part I evidence was reviewed showing that the frequencies of so called "spontaneous" central nervous rhythms may serve as a relative measure of the tissue's metabolism. The μ values for alpha brain wave frequencies are shifted by syphilitic infection from 8 to 11 to 16 thousand calories. Since we have shown that endogenous brain iron is partially "precipitated" out as a result of the infection, we might expect an iron catalyzed step (such as that of the cytochrome-cytochrome oxidase system) to become the rate-determining link resulting in a μ of 16,000 calories in advanced paresis. In Part II we have shown that by reducing the activity of some of the cytochrome-cytochrome oxidase with cyanide we get a μ of 16,000 calories and we have presented evidence that the value of 11,000 calories, so often characteristic of tissue oxidations, corresponds to succino-dehydrogenase as the rate-controlling factor for O_2 consumption. So far no certain statements can be made concerning the enzyme system corresponding to 8000 calories, although Graubard, Hoagland, and Foxhall have preliminary evidence indicating that this may correspond to copper catalysis in certain reactions. Copper is known to be an important constituent of widely distributed oxidases. It is present in most animal cells including the brain (Elvehjem, 1935).

Quastel (1939) in a recent review summarizes evidence indicating that glucose is probably the only substrate normally involved in brain oxidations. The importance of the rôle of the C_4 dicarboxylic acids, including the succinate-fumarate system has been stressed by Szent-Györgyi (1937) in connection with glucose oxidation in tissues.

Torrès (1935) finds that cyanide inhibits the respiration of rat brain 97.5 per cent. Quastel (1939) writes, "The large inhibitory effect of cyanide on brain respiration points to the probability that the respiration proceeds largely through the cytochrome-cytochrome oxidase system. The small cyanide-insensitive part of the respiration may be due to the activity of a flavine system."

We do not wish to imply that 16,000 ± calories is necessarily always to be found associated with the cytochrome-cytochrome oxidase system. If cytochromes *a*, *b*, and *c* act sequentially along with their sequential oxidase (possibly copper-containing) step, this would at once suggest the possibility of several other μ values as temperature characteristics for this system. Approximately 16,000 calories was found under the conditions of our experiments. The prominence of this value in physiological systems together with other matters discussed above indicates that its occurrence is due to the cytochrome system acting as the slow link in a variety of biological oxidations under conditions in which this value is characteristic for the iron-containing cytochrome-cytochrome oxidase system as a whole.

SUMMARY

1. Iron spicules found in the brains of general paretic patients are formed from endogenous brain iron normally present in another form. This supports our earlier view that the μ value of 16,000 obtained in advanced paretics for alpha brain wave frequencies as a measure of cortical respiration comes about from the slowing of an iron catalyzed link in cortical respiration such as would result from the reduction of available cytochrome and its oxidase, thus making this step a chemical pacemaker.

2. To test the basic theory of chemical pacemakers, a study was made of the succinate-fumarate enzyme system containing succino-dehydrogenase and cytochrome-cytochrome oxidase acting sequentially.

3. The μ value for the unpoisoned system is 11,200 ± 200 calories.

4. According to theory, the addition of a *critical* amount of cyanide known to be a specific poison of the cytochrome-cytochrome oxidase system (and not of the dehydrogenase) should shift the μ cleanly to 16,000 calories, and it does.

5. According to theory, selenite, a specific poison for the dehydrogenase, should stop all respiration without shifting the μ. This also is found to be the case.

6. The theory also predicts that if the μ is shifted from 11,000 ± to 16,000 ± by cyanide, the subsequent addition of a *critical* amount of selenite should shift the μ back again to 11,000 ± calories, and this is found to occur.

7. It is concluded that approximately 11,000 calories is the energy of activation of the succino-dehydrogenase-catalyzed step and 16,000 calories is that for the cytochrome-cytochrome oxidase-catalyzed step. These two values are encountered more frequently than any others in physiological systems. It is to be recalled that a shift of μ for alpha brain wave frequencies from 11,000 to 16,000 calories occurs in the course of advancing syphilitic brain infection and is accompanied by a change in form of brain iron.

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THEORY AND MEASUREMENT OF VISUAL MECHANISMS

III. ΔI As a Function of Area, Intensity, and Wave-length, for Monocular and Binocular Stimulation

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I

An increase in the size (A) of a uniformly illuminated retinal light image usually occasions a decrease in the amount of light ($= I \times t$) required to elicit a threshold response (Aubert, 1865; Riccò, 1877; Abney, 1897; Piper, 1903; Henius, 1909; Fujita, 1909; and Piéron, 1920*a*, *b*). With exposure time as parameter, the intensity of the threshold stimulus (herein labelled ΔI_0) has been found empirically to be, nearly enough, but with certain qualifications and restrictions, a declining power function of the size of the retinal image (Abney, 1897; Abney and Watson, 1916; Wald, 1937–38), at least over a limited range of A and of ΔI_0:

$$\log \Delta I_0 = -z \log A + C; \tag{1}$$

ΔI_0 is the intensity of the threshold stimulus (*i.e.*, for this case, $\bar{I}_2 - I_1 = \bar{I}_2 - 0 = \Delta I_0$, in photons); A is the size of the retinal image (in square millimeters, or in degrees visual angle for a symmetrical figure—up to *ca.* 5°); and z and C are constants. The value of z, however, also proves to be a systematic function of particular physiological variables. Equation (1) then assumes the form of a general exponential

$$\Delta I_0 = C'A^{-z} \tag{2}$$

in which z is no longer to be regarded as sensibly constant; instead it is to be treated as a function of one or more dimensions of the retinal light image, so that $z = f$ (I_1, ΔI, A, retinal location, form of test patch, exposure time, ...).

Attempts have been made to account for the properties of threshold data primarily in terms of the assumption that their quantitative features are determined by events at or in the retinal receptors (*cf.* von Kries, 1911; 1929; Hecht, 1937). A variety of considerations leads us to reject this assumption (Crozier, 1936; Crozier and Holway, 1938–39 *b*, *etc.*). Func-

tional properties of the central nervous system provide a more comprehensive basis for the interpretation of the data of intensive discrimination.

Practically all theories of absolute and relative threshold responses have invoked the constant quantity or constant number concept, in some form (*cf.* Weber, 1834; Wald, 1937–38). The assumption involves serious difficulties, and its necessity has never been demonstrated. If used with reference to central nervous properties, the assumption leads to the proposition that the visual minimum perceptible depends upon the eventuation in the nervous system of a definite number of nervous impulses per unit time, or of a certain density of such impulses (*i.e.*, number/unit time/unit volume). The weakness in the traditional mode of using this general conception lies in taking it for granted that this "definite number" is fixed and constant; this leads to the assumptions (*a*) that threshold brilliance increments are equivalent, (*b*) that just discriminable steps in sensorial effect are equal. Over a certain range of intensities this is approximately true, but even the approximation is illusory (Crozier, 1936; Crozier and Holway, 1938–39 *a*, *b*, *etc.*). When appeal is made to "number of excitatory impulses per unit time" we, however, need not and should not be restricted to the notion that for threshold or other intensive discrimination there obtains the requirement of *constancy* in this number at all levels of intensity, or of area, or of retinal location. The statistical mechanism of discriminatory performance definitely forbids the restriction of constancy (Crozier, 1936). The manner in which magnitude of sensory effect E is really related to I, and which determines the nature of ΔE and of ΔI as a function of I, is to be ascertained by means of considerations arising as a consequence of the measured properties of ΔI.

Qualitatively we may assume that the number of central nervous units or elements of excitation (*i.e.*, the number per unit time) increases with increase of area in the retinal image, and in addition their density with increase of intensity, and with increase of exposure time (over a limited range). For present purposes we need not attempt to specify the precise law of this increase. The excitation required for threshold visual effect must, however, be supposed to involve two factors, (*a*) the number of neurons affected and (*b*) the frequency of discharge of impulses in each. The basic supposition here is of course that the rules for activity in single peripheral nerve fibers apply also to central nervous units (Lucas, 1917, *etc.*).

Much has yet to be made out as to the quantitative relations obtaining between the structural elements at various levels in the human visual apparatus (Poljak, 1935; Østerberg, 1935). But it is safe to presume that,

in general, when the number of active central elements is increased by enlarging the size of the light image on the retina, a smaller density of impulses per unit illuminated area of retina should be required to eventuate the frequency of impulses necessary for sensorial discrimination, with other things constant. The relations between critical flash frequency and flash illumination for response to flicker illustrate these considerations (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*).

The rôle of the spatial distribution of sensitivities and effectivenesses of the retinal elements and the nerve fibers involved is a separate problem. Whatever the form of this distribution, even if it be one of entire uniformity, the frequency factor must enter, and thus influence the intensity (flux/area) required to produce the necessary number of impulses per unit area. The same type of reasoning applies for the analysis of the time intensity functions (*cf.* data of Graham and Margaria, 1935). Moreover, it is clearly required that ΔI_0 for binocular stimulation must on this basis be *less* than that for monocular presentation. This has sometimes been denied (*cf.* Graham, 1930), but is substantiated by tests in which proper provision has been made to insure binocular accommodated fixation.

The properties of the *minimum discriminable*, ΔI, like those of the *minimum perceptible* (ΔI_0), can be accounted for in the same terms. In general, ΔI (for a given I_1), decreases as the size of the retinal light image is increased (Lasareff, 1911; Heinz and Lippay, 1928; Cobb and Moss, 1928; Steinhardt, 1936–37; Holway and Hurvich, 1938). No *homogeneous* data (Crozier, 1936) have been available, however, for defining the relations between ΔI and A for monocular and binocular excitation at various intensity levels. The present paper contains such data, with exposure time, intensity, and several wave-lengths as parameters. We consider here chiefly measurements made with two observers for which the excitability functions of each of the two eyes are very nearly the same. Results with an observer for whom this is not the case are important for the general theory, and will be treated separately.

II

Apparatus and Procedure

Measurements of ΔI ($= \bar{I}_2 - I_1$) as a function of the size of the mean visual angle were made at various intensities and wave-lengths for both monocular and binocular stimulation. For monocular excitation a uniformly illuminated, rectangular light image was projected upon the retina, centered at the fovea. Six areas were used. During any single sitting, wave-length (λ), intensity (I_1), and exposure time were parameters. Before each sitting, the observer dark-adapted for 20 minutes. The experiments began with the weakest (photopic) intensity and the smallest area. At the

beginning of a sitting, the observer adapted to the prevailing intensity for about 30 seconds. Then the experimenter added light to I_1 at a constant rate for log I, until a just noticeable increase in brilliance was reported. Five ΔI measurements were taken in this manner for the smallest area in each series. The size of the light image was then doubled, while I_1, λ, and other conditions remained unchanged. When five measurements had been made for this area, the image was again doubled in size. This practice was continued until five measurements had been secured for each of the six areas at the lowest intensity. The same procedure was then employed for an intensity about 10 times as great as the preceding I_1. Thus, with respect to both area (A) and intensity (I_1), the order of securing the measurements was always in the direction of increase.

The method of presentation of the conditions under which judgment of just noticeable intensive differences is to be made has of course a decided influence upon the magnitude of ΔI for a given I_1, as is well known. The procedure adopted for the present experiments was chosen to avoid as far as possible the effect of the presence of a fixed "surround." It is known (Aubert, 1876; Cobb, 1916; Guild, 1932) that the presence of an illuminated area surrounding the adjusted illumination of the test-patch, particularly at higher levels of illumination, reduces ΔI; at minimal and sub-threshold illuminations ΔI is *increased* (unpublished data). The result at high illuminations is not altogether due to the blotting out of internal reflections in the eye-piece of the observing instrument, although this is probably an additional factor in the choice of a surround in certain cases. There remains the difficulty, however, of deciding *how* large and *how* intense a surround to employ, and whether it should be of constant intensity, or equal or proportional to I_1. Decision on these points cannot be based on agreements of the induced properties of ΔI with the requirements of a particular theoretical interpretation of the dependence of ΔI upon I_1; the influence of the surround upon the properties of ΔI is known to be a function of its area and intensity, as well as of the level of I_1. The best solution is not to employ a fixed surround at all.

The same procedure was adopted for the binocular measurements. The observers adapted in the dark for 20 minutes or 45 minutes, depending on the nature of the experiment (*vide infra*). Starting at the lowest value of I_1, and the smallest area, ΔI measurements were taken at all areas for the weakest intensity used for white light or for a given wave-length. Then I_1 was increased tenfold and ΔI values were secured for all areas in order of increasing magnitude.

Only light of a given wave-length composition was used during a single sitting. Hence, for any given wave-length and intensity specifications, the measurements are homogeneous for the determination of the relation between ΔI and A, the angular size of the retinal image.

Fig. 1 gives a simplified plan-view of the apparatus. This instrument, a visual discriminometer, has been described in some detail (Crozier and Holway, 1938–39*a*). S_0 is the primary light source,—in this case a flat ribbon filament, especially designed, operating at 6 volts and 30 amps. The following controls served to eliminate fluctuations in emission due to variations in the line. The current taken from the line passes through a GR Variac (an auto-transformer with continuously adjustable output) to two transformers (capacity = 20 amps., 6 volts) in parallel, and thence through a 30 amp. fuse and a Westinghouse No. 37 ammeter to the lamp. With this arrangement, variations in current as small as 0.5 of 1 per cent can be detected immediately by the experimenter and adjusted at the variable transformer. The lamp was set in operation

for at least 20 minutes before each experiment. (About 15 minutes are required before a steady level of current consumption is reached.) A continuous air blast through the lamp-house insures constant emission after this period of time, provided of course that fluctuations do not occur in the power line. Only twice during our experiments have

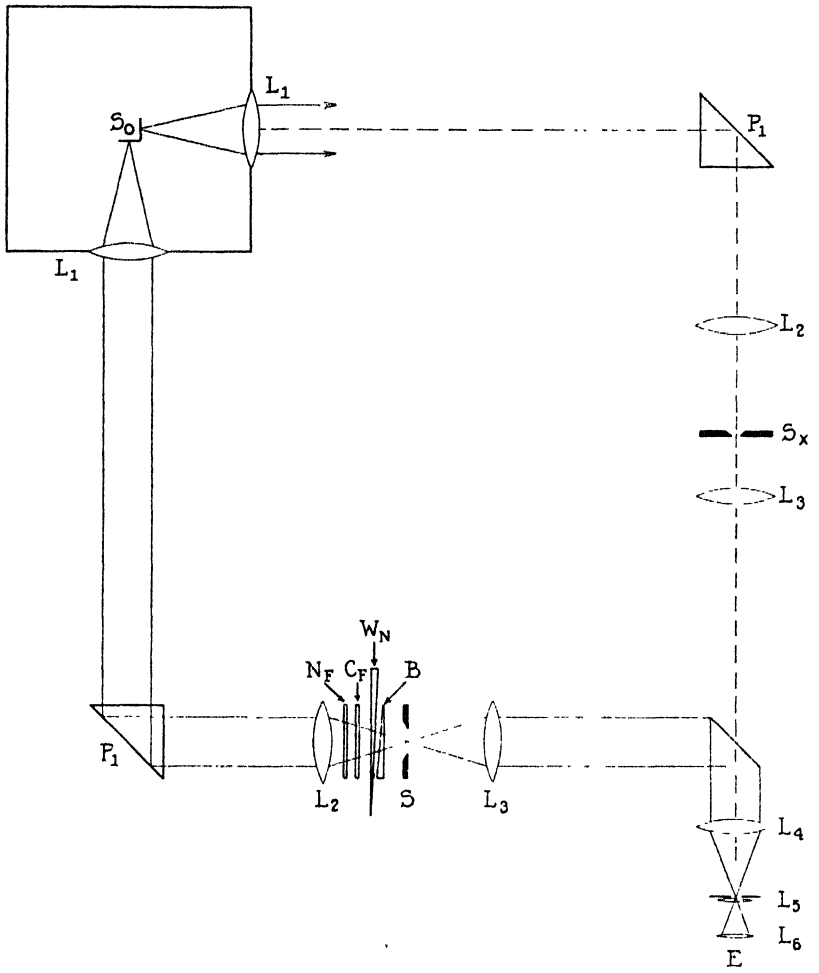

FIG. 1. Schematic plan view of the optical arrangement used in the monocular experiments. For binocular stimulation, a binocular head with matched oculars is substituted for *O*. See text.

we found it necessary to readjust the power supply at the secondary coil of the variable transformer to correct for variations in the line. These fluctuations, however, were of sufficient magnitude to justify our precautions (as a 10 per cent change in current would occasion slightly more than one log unit change in intrinsic brightness). Through-

out the experiments, the current was maintained at 30 amps. The resulting emission gives rise to a brilliant 'snow white' impression.

Light emitted at the constant source S_0 passes through the large quartz condensing lenses, L_1, which are located at a distance equal to their focal lengths from the plane of the flat ribbon filament. Parallel light transmitted along the axis of the collimators is totally reflected 90° by the prisms at P_1. The lenses L_2 place a uniformly illuminated image of S_0 in the plane of the bilateral slits, S. One of the slits contained the fixation point; the other served as variable aperture. Capstan screws regulate the size of the aperture defined by these slit knife-edges. L_3, situated at a distance from S equal to its focal length, sends parallel bundles of light to the front-surfaced mirror (chrom-aluminized by sputter) which deflects the beam 90°. L_4 forms an image of S in the plane of the stop A and the flat surface of the field lens L_5. L_6 is the eye-piece (mag. = 15 ×). With the eye at the exit pupil of the ocular, an observer sees a rectangular field of constant height; the fixation dot lies in the center of the field. All lenses are achromatic. A Bausch and Lomb combination chin-rest and head-support is used at E.

For binocular stimulation, a binocular head was substituted for the ocular, O. Here, right angle prisms divide the beam and two optically identical images of the aperture S are located in the plane of surfaces of the field-lenses in the matched oculars. The proper interocular distance is obtained by adjusting a graduated drum. The slight differences in accommodation which exist for practically all observers are corrected by turning the spiral tube in which one ocular is mounted.

Neutral tint filters inserted at F regulate the fixed values of I_1. The transmission of these filters for white light was determined with a König-Martens polarization photometer. We are under obligation to Dr. C. P. Winsor for his collaboration in these measurements. Four measurements were made in each quadrant,—a total of sixteen in all. Failure to use all quadrants invariably leaves the transmission coefficient with a constant error and thus defeats the chief purpose of the measurements, which is to eliminate constant errors. The probable error of the mean of these measurements is usually less than 0.5 of 1 per cent. Frequently, however, it may be much less; photometric results, even for different observers, may differ only in the fourth place of the mantissa when the transmission, or density, is expressed in logarithmic units.

For wave-length composition as parameter, Wratten color filters Nos. 71A, 74, and 47 were used. The wave-length composition for each filter was determined by a photoelectric spectrophotometer (Hardy, 1929; 1935). These filters transmit frequency "bands," not lines. The spectral distribution of each color filter used is shown in Fig. 2. The transmission coefficients for the light emitted by S_0, however, were measured with a Leeds and Northrup galvanometer (sensitivity = 7 mm./μv at one meter) in series with a large surface Moll thermopile. Even though the lenses, L_1, are quartz, some heat rays can be detected at E (Fig. 1) at highest intensities. It was therefore necessary to use an additional heat filter. A disc of colloidal gold (suspended in glass) inserted in the instrument at L_1 served this purpose adequately. The effectiveness of this arrangement was verified by further measurements made with a rock-salt crystal in front of the thermopile at E. The transmission coefficients of the neutral tint wedges (and filters) were also determined in this manner.

The diameter of the eye-ring was less than 2 mm. and served as an effective artificial pupil. Corrections (over-all) were made for the probable losses in transmission suffered through absorption in the ocular media (*cf.* Roggenbau and Wetthauer, 1927; Ludvigh

and McCarthy, 1938). The results, all in energy units, were converted into millilamberts by means of a single binocular match (white light), using a Macbeth illuminometer and standard test-plate. Although these average corrections are imperfect for any given eye, they are in the proper direction and are consequently better than no correction at all. Finally, all intensity values were expressed in terms of retinal illumination, as photons (Troland, 1918).

The shape of the aperture was rectangular at S, and the trace of the light disturbance cut by a plane located at right angles to the optic axis at the retina is similar in form. The angular *height* of this image was constant (= 20.8°). Area was regulated by lateral movement of the horizontal knife-edges at S. The angle subtended by the retinal light image at the principal point of the eye was measured with the aid of an ocular micrometer. The graticule was placed in the plane of the circular stop at the field lens and the screws controlling the lateral width of the slit at S were calibrated so as to read directly in terms of the visual angle. Area measurements can of course be expressed in square millimeters on the retina. For large areas, however, it is usually more convenient to employ angular readings and all our measurements are tabulated in degrees of visual angle.

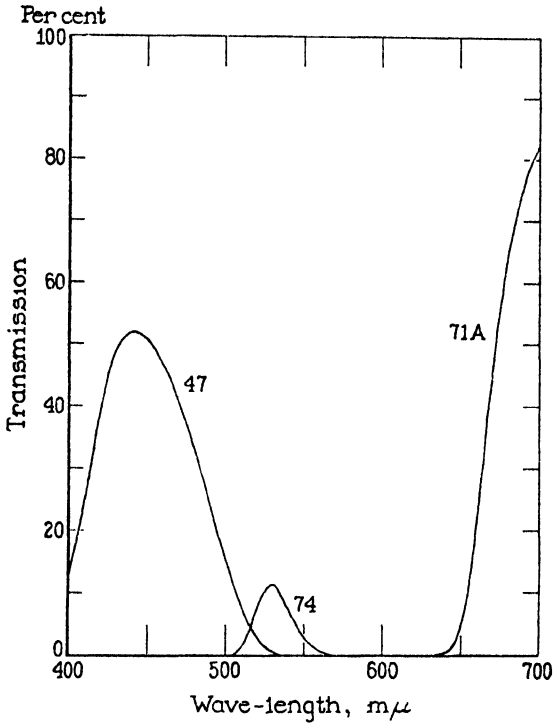

FIG. 2. Showing the wave-length *vs.* intensity distribution of the color filters used in the present experiments. The determination of these curves was made with a photoelectric spectrophotometer (Hardy, 1929; 1935).

The observer was instructed to focus upon the tiny (red) fixation point located in the center of the rectangular light image. The position of this point was controlled at S_x. The stimulus was fixated for about 30 seconds before ΔI was added to I_1.

III

Monocular Measurements, White Light

For white light, monocular measurements were secured at six areas of different size at each of six levels of intensity, I_1. The results for one

observer (A. H. H.) are shown in Table I. At any level of I_1 the data are homogeneous with respect to area. The vertical height of the image was constant at a visual angle of 20.8°, and the A' values in Table I represent the visual angle subtended at the eye by the lateral separation between the vertical edges of the slits in image space. I_1 is the prevailing intensity. I_1 was adjusted in exact steps by suitable adjustments of the wedge W_n (Fig. 1) in terms of the calibrations of the decimal filters N_f. Exposure time (= 30 seconds) and wave-length composition are parameters. Each

TABLE I

White light: homogeneous results for ΔI as a function of A' and I_1; monocular; I_1 is the standard intensity, in photons; A', the angular width of the light image on the retina—the angular height of the image (relaxed accommodation) was constant, = 20.8°. Each ΔI entry is an average of five measurements; $\sigma_{\Delta I}$ is the root-mean-square variation of a single observation. A.H.H.; right eye. See Fig. 3.

Visual Angle, A' Degrees

log I_1, *photons*		0.4°	0.8°	1.6°	3.2°	6.4°	12.8°
$\bar{2}.006$	log ΔI_m	$\bar{3}.621$	$\bar{3}.587$	$\bar{3}.445$	$\bar{3}.365$	$\bar{3}.233$	$\bar{3}.204$
	log $\sigma_{1\Delta I}$	($\bar{4}.718$)	($\bar{4}.544$)	($\bar{4}.471$)	($\bar{4}.410$)	($\bar{4}.317$)	($\bar{4}.321$)
$\bar{1}.006$	log ΔI_m	$\bar{2}.400$	$\bar{2}.254$	$\bar{2}.203$	$\bar{2}.181$	$\bar{2}.032$	$\bar{3}.999$
	log $\sigma_{1\Delta I}$	($\bar{3}.413$)	($\bar{3}.344$)	($\bar{3}.306$)	($\bar{3}.207$)	($\bar{3}.101$)	($\bar{3}.015$)
0.006	log ΔI_m	$\bar{1}.282$	$\bar{1}.157$	$\bar{1}.078$	$\bar{1}.063$	$\bar{2}.923$	$\bar{2}.778$
	log $\sigma_{1\Delta I}$	($\bar{2}.293$)	($\bar{2}.160$)	($\bar{2}.105$)	($\bar{3}.994$)	($\bar{3}.977$)	($\bar{3}.790$)
1.006	log ΔI_m	0.118	$\bar{1}.992$	0.048	$\bar{1}.999$	$\bar{1}.800$	$\bar{1}.655$
	log $\sigma_{1\Delta I}$	($\bar{1}.107$)	($\bar{2}.990$)	($\bar{1}.131$)	($\bar{1}.004$)	($\bar{2}.903$)	($\bar{2}.707$)
2.006	log ΔI_m	1.088	0.963	0.880	0.902	0.788	0.601
	log $\sigma_{1\Delta I}$	(0.091)	(0.002)	($\bar{1}.913$)	($\bar{1}.966$)	($\bar{1}.810$)	($\bar{1}.675$)
3.006	log ΔI_m	2.091	2.000	1.947	1.834	1.754	1.639
	log $\sigma_{1\Delta I}$	(1.135)	(1.011)	(0.923)	(0.914)	(0.799)	(0.708)

ΔI_m entry is an average of five measurements. Associated with each ΔI_m is the measure of dispersion, $\sigma_{1\Delta I}$, the root-mean-square deviation of a single observation. For any fixed value of I_1, both ΔI_m and $\sigma_{1\Delta I}$ are seen to vary inversely with the size of the retinal light image.

These ΔI_m data are plotted in Fig. 3. The coordinates are spaced logarithmically. Units of area (A, square degrees) are used. Each plotted point is an average of five measurements. The solid lines were fitted by the method of averages, and are described by

$$\log \Delta I = -Z \log A + C, \text{ as in (1)} \tag{3}$$

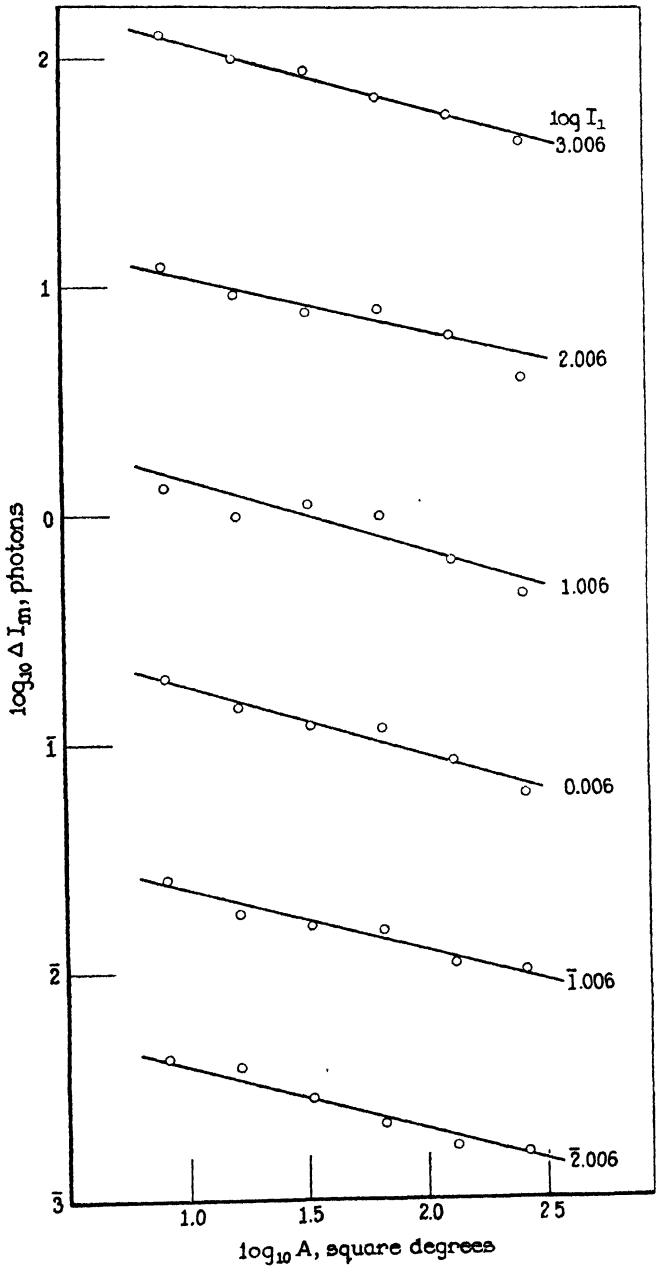

FIG. 3. Dependence of ΔI_m on A; homogeneous data for white light, monocular excitation (Table I). Intensity I_1 as parameter. The lines adjusted are for $\log_{10} \Delta I_m = -Z \log_{10} A - C'$, with $Z = 0.267$; A is in *area* units (square degrees). The maximum departure is less than $4 \times \sigma_{\Delta I_m}$.

where ΔI is the added intensity $(I_2 - I_1)$ required for the index response. In terms of the measure of sensitivity, $1/\Delta I$ at each level of I_1,

$$\log (1/\Delta I) = Z \log A - C. \tag{4}$$

Fitting by the method of averages, mean $Z = 0.267$ when logarithms of *area* and of ΔI are taken to base 10. If $\log A$ is taken to base 2, the mean slope Z is 0.081. This signifies that, for the conditions considered, an approximately twofold increase in area of the retinal image reduces ΔI_m by a factor of 1.205 arithmetic units. The S.D. of the six values of Z from the slopes in Fig. 3 is only $\sigma_1 = 0.0022 \log_{10}$ units. The (monocular) values obtained with colored lights (section V) are also of just this order, although a little smaller. The ratio for doubling area on one retina is therefore definitely *less* than that obtained for the comparison of monocular and binocular thresholds with a *small* fixed area variously located on the retina (Crozier and Holway, 1938–39 *b*), namely 1.4.

IV

The Area Function

The form of equation (4) is the same as that for ΔI_0 in equation (2). Its derivation may be obtained without specific assumptions as to the nature of the threshold of excitability. Such assumptions are to be avoided if possible, since the discovery of the mechanism of threshold effects is one of the objectives of inquiry, and preferably one should not be theoretically committed in advance.

At any level of I_1 a change in the conditions and method of presentation can lead to a change in ΔI. The brilliance increase necessary for the recognition of ΔI can be produced by suitable increase of the excitable area, as both theory and qualitative tests indicate, and our own observations. This implies that, for a given level of I_1, the excitability $(1/\Delta I)$ measures the same kind of a property of the capacity for reacting performance based on recognition of intensive difference as is also measurable by area A. These two measures of the capacity for excitation vary together and directly, although not rectilinearly. The multiplication of the units of one of these measures by the appropriate factor $d(1/\Delta I)/dA$ giving the rate of change of $1/\Delta I$ per unit change of A gives the dimensionally correct expression

$$1/\Delta I = k\, A\, d(1/\Delta I)/dA \tag{5}$$

Rearranging,

$$d(1/\Delta I)/(1/\Delta I) = Z\, dA/A,$$

or

$$\log \Delta I = -Z \log A + C,$$

as already found in empirical equation (1, 4). If over a particular range the factor k, (or $1/Z$), is really constant, and independent of I_1 and of A, we can expect this equation to hold independently of I_1, A, exposure time, and the monocular or binocular mode of presentation; Z should be slightly less for colored light than for white, for reasons considered subsequently (section VII). The dimensional constant C has the meaning of a ΔI times an A.

An equation of the general form of (1) and (5) was derived by Wald (1937–38) for visual thresholds and area, using the conceptions that (1) there is required a constant number of excited elements at the threshold; and (2) that the excitability is statistically distributed in a homogeneous retinal area, in a manner given by a probability summation. In the symbols of our equation (1) and (5), Wald's formula is $-\log \Delta I_0 = k \log (A - n_t) + C$, where n_t is the threshold number of excited elements (*i.e.*, the "active area"). There are certain difficulties with this expression, and its derivation. Obviously, when $A = n_t$ we have

$$\log 1/\Delta I_0 = -\infty, \text{ or } \Delta I_0 = \infty;$$

this contradicts the assumption that ΔI_0 is the intensity required to excite n_t elements. This is the assumption used in deriving the equation, A being really a measure of the probability of finding n_t elements under the conditions of presentation, and essentially in terms of *number* (frequency of encounter) of elements. The argument cannot hold for the application of the equation to the case of $\Delta I = \bar{I}_2 - I_1$, the differential threshold, for similar reasons. If equation (4) is solved for the integration constant C it is apparent that

$$C = \log C' = \log (\Delta I)\,(A^Z)$$

and

$$C' = (\Delta I)\,(A^Z),$$

or

$$C'/A^{(Z-1)} = \Delta I \cdot A.$$

The integration constant has therefore the dimensions of *intensity* times the *area* raised to a power. If we wish to define $A_1 = n_t$ as the *unit* area (in terms of n) capable of being excited in terms of ΔI_0 or of ΔI, then we can write

$$C'/M = C'' = \Delta I_1$$

where M is given by

$$M A_1^Z = A^Z$$

and ΔI_1 is the threshold intensity increment for the response with the elementary "area." Consequently, on the assumption that Z is a constant, the use of the "constant quantity" or "constant number excited" idea really requires that the correction for the elementary, active area (and the threshold for this area) be carried in the terminal constant C of equation (4); it should not be corrected for, as in Wald's treatment, by subtracting A_1 (or its equivalent n_t) from A, but takes the form of a multiplier. The theoretical reason for doing this is of course that if (in terms of the fixed quantity concept) A determines the probability of finding n_t, this probability is *also* measured by ΔI; the dimensional constant C in equation (4) must include both A *and* ΔI. The descriptive power is not improved by subtracting a constant from A. What the equation says is that the threshold excitability per unit area $(1/\Delta I \cdot A)$ is proportional (inversely) to a fractional power of the area.

When C is evaluated by taking $A = 10$ units (or any other constant value),

$$C = \log C_1 = \log \Delta I \text{ with } A \text{ fixed,}$$

C should follow the same law as $\log \Delta I$ when I_1 is varied; examination of the data (Fig. 3, *etc.*) shows that this is the case; C *vs.* $\log I_1$, with A fixed, gives curves reflecting exactly the behavior of $\log \Delta I$, including the fact that (*cf.* section IX) binocular C is lower than the monocular.

The condition for constancy of Z is, from (5), that

$$\frac{d(1/\Delta I)}{dA} = Z/A \cdot \Delta I \tag{6}$$

The product $A \cdot \Delta I$ is directly proportional to the increase of energy (flux per unit time and per unit area, multiplied by area) required to produce recognition of increase of brilliance; this cannot be constant if equation (6) is empirically valid, as it is shown to be as a very fair approximation. It follows from (6) that if Z is constant when A is increased, the added energy required for recognition of increase of brilliance is inversely proportional to the (average) increase of excitability per unit increase of area.

This entirely reasonable proposition can obtain only if the retinal field concerned, and its central representation, is of approximately uniform excitability over its whole extent, at the level of discriminatory response. This condition may well obtain for sufficiently large retinal fields symmetrically centered at the fovea; for an increased area, ΔI_0 and ΔI are then

less than for a smaller area, but the appearance of the smaller area included in the larger is nevertheless of the same brightness as for the rest of the field at the differential threshold for the larger area; the smaller area, of lower intrinsic excitability, when tested by itself, is not seen as such any more than the blind spot is. The larger area as a whole behaves as if of uniform excitability, a fact easily understood on the basis that the measurable properties of intensive experience are determined centrally rather than peripherally at the retina. From this standpoint only the analytical use of "average excitability" has a real physical basis.

TABLE II

Area increased by enlargement toward the fovea, the outer margin of the 20.8° high test patch being kept at 12.8° from the center of the fovea. A.H.H., right eye; white light; 20 minutes dark adaptation, 30 seconds light adaptation; ΔI_m is the mean of five observations; $\sigma_{1_{\Delta I}}$ is the dispersion of these; I_1 is the initial intensity; other conditions as described in the text; A' is the angular breadth of the test patch. Plotted in Figs. 4 and 5.

		A', *degrees*					
log I_1, *photons*		0.4°	0.8°	1.6°	3.2°	6.4°	12.8°
$\bar{1}$.680	log ΔI_m	**$\bar{2}$.884**	**$\bar{2}$.530**	**$\bar{2}$.512**	**$\bar{2}$.341**	**$\bar{2}$.227**	**$\bar{2}$.071**
	log $\sigma_{1_{\Delta I}}$	($\bar{3}$.921)	($\bar{3}$.665)	($\bar{3}$.416)	($\bar{3}$.305)	($\bar{3}$.015)	($\bar{3}$.122)
0.680	log ΔI_m	**$\bar{1}$.619**	**$\bar{1}$.481**	**$\bar{1}$.340**	**$\bar{1}$.074**	**$\bar{2}$.934**	**$\bar{2}$.687**
	log $\sigma_{1_{\Delta I}}$	($\bar{2}$.703)	($\bar{2}$.509)	($\bar{2}$.532)	(2.111)	($\bar{2}$.930)	($\bar{2}$.004)
1.680	log ΔI_m	**0.611**	**0.533**	**0.159**	**0.163**	**$\bar{1}$.921**	**$\bar{1}$.830**
	log $\sigma_{1_{\Delta I}}$	($\bar{1}$.628)	($\bar{1}$.480)	($\bar{1}$.062)	($\bar{1}$.205)	($\bar{1}$.005)	($\bar{1}$.070)
2.680	log ΔI_m	**1.583**	**1.599**	**1.274**	**1.013**	**0.938**	**0.825**
	log $\sigma_{1_{\Delta I}}$	(0.605)	(0.591)	(0.288)	($\bar{1}$.994)	(0.107)	($\bar{1}$.738)

Under these conditions Z is found to be nearly independent of A, I_1, and wave-length, and also of exposure time (*cf.* Crozier and Holway, in preparation). So also in certain other types of intensive discrimination, such as that (Crozier, 1935 36) concerned in measurements of visual acuity (Freeman, 1932; 1936), the same formulation is applicable. An arrangement is easily obtained, however, in which Z is constant but has a numerical value different from those already seen. It is not independent of the *shape* of the test patch. The rectangular image as already used (Table I) is in the present test excentrically located, and the edge farthest from the fovea is kept at a fixed position while, for enlargement of the area, the inner edge is moved closer to the fovea. With this arrangement an increase of the area necessarily involves a progressively greater change in the rate of addition

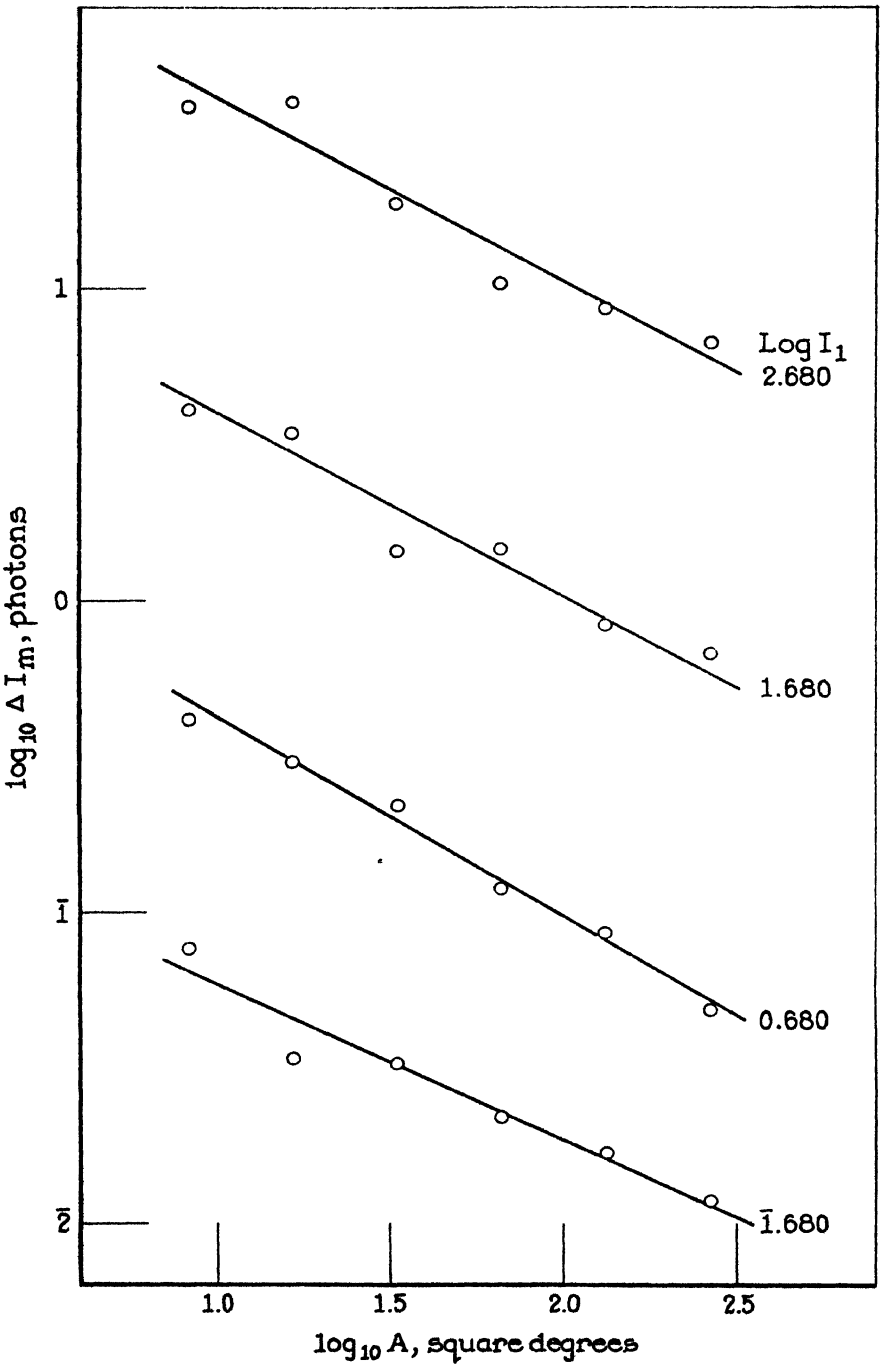

FIG. 4. Data in Table II; see text.

of excitable elements. It is necessary to remember that when area is increased in this manner the edge advancing toward the fovea in successive tests includes at each step regions of the retina for which the absolute excitatory thresholds (ΔI_0) per unit area are successively higher; but each of the doubling steps embraces a larger increment of area; the additional

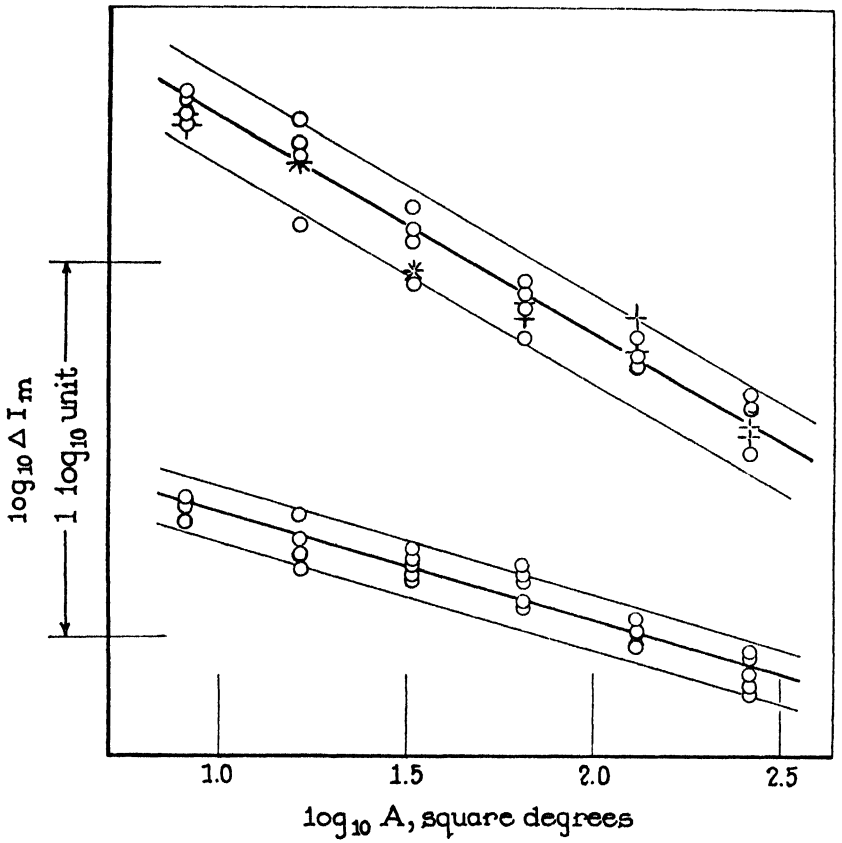

FIG. 5. Data of Fig. 3 (lower plot) and of Fig. 4 (upper plot; with figures from two other sets, not tabulated, given as crosses), brought together at the midpoint for comparison of slopes. The slope (Z in equation (2)) is independent of ΔI_m, and thus of I_1, but is dependent on the manner in which retinal area is increased; see text.

fact that the *differential* excitatory threshold (ΔI, for finite I_1) per unit area declines with approach to the fovea shows that appeal must be made to the changing ratio of numbers of receptor end organs to optic nerve fibers if these two excitability phenomena are to be explained. It must be expected, in terms of the introductory discussion (section I), that in such

an experiment the value of Z, if constant, must be *higher* than for tests of the type considered in Table I. For one of the same observers, data are given in Table II. It is apparent (Fig. 4) that equation (5) gives a very fair description of these data, and that the value of Z is indeed much greater, being 0.591 as compared with 0.267 in Fig. 3. Each doubling of the area on the average reduces ΔI by a factor of 1.52 (in the *same* arithmetic units), rather than by 1.21 as in Table I. Fig. 5 shows that the scatter of the determinations, with all the sets of points brought for comparison to the same log ΔI ordinate at the midpoint, is not too great; it is a little greater, understandably, in the experiment of Table II, since there fixation was outside the test patch. (The series of Table I show (Fig. 5) a certain consistency of bowing upward, which is not apparent in those for Table II and is probably not significant.)

In the discussion of these data it has thus far been accepted that increase of *area* is the significant factor in determining change in $1/\Delta I$ (with I_1 fixed = 0 or some finite value). We have to test this notion. The extent to which it is legitimate will necessarily be restricted by the geometrical pattern of neural organization in the retina. This is in the main on a plan with radial symmetry about the fovea (Østerberg, 1935, *etc.*). When a test patch is used of such form that increase of its area involves radially symmetrical enlargement, as with a circle or square of fixed center, it cannot be told for small ranges of area whether *area* or a linear *visual angle* is the governing feature (*cf.* Abney and Watson, 1916). The exponent Z will of course differ in these two relations, by a factor of 2, since log A will be equal to 2 log D + *const.* (where D is a linear dimension). This was one reason for our selection of rectangular test fields. With constant vertical height of field, Z is of course the same whether log *visual angle* of breadth or log *area* is used. But by employing two or more sets of rectangular test fields of constant but different heights (section VI) we can discover whether *area* or *angular breadth* gives the proper units for analysis.

V

Power functions, such as represented by equation (5), are rather frequently found to be serviceable for the formulation of properties of natural phenomena. And not infrequently the exponent in such a formulation is fractional, not integral, as with our Z.

In dimensional analysis it is recognized that for measurements in which secondary or derived units are used the fundamental units necessarily enter as products of integral *powers* of the primary variables. It has also been recognized (by some at least) that the occurrence of *fractional* powers is

eminently puzzling. If, however, we are dealing with the relation between two indirect, derived ("secondary") measures of the same fundamental attribute, the occurrence of a power function for the relationship between them may well be expected; and there is every reason to find that the exponent is frequently not an integer. In deriving equation (5) we have taken the view that the capacity for excitation is a function of the area A. Presumably this is in some fashion to be expressed in terms of number of potentially excitable central elements and their interconnections. A is then to be taken as a function of products of powers of these elementary dimensions (*cf.* Bridgman, 1922). The deduction of the nature of these elementary dimensions (factors) is of course the essential problem. Since it must be approached indirectly, we are forced to use other, additional modes of measuring the capacity for excitation. We might use exposure time, but ΔI is experimentally more flexible. The same fundamental excitability factors are involved. As different measures of the excitability, we have A and ΔI; for simplicity in illustration we may assume that each is a function of merely the number of potentially excitable elements, raised to a power. The exponents need not be the same. In this case the experimentally found relation of ΔI to A would be given by a power function, and the exponent could have almost any finite value; particularly when it is recognized that the "number of elements" is (in view of the fact of central summation - *cf.* Crozier and Holway, 1938–39) measured by a complex quantity.

When one speaks of a property or capacity of biological performance as being measured in "secondary units" which follow a law determined by the operation of "fundamental" or primary units, a certain vagueness is necessarily introduced by one's inability to write down the list of primary units necessarily involved. This has probably been responsible for the view (Bridgman, 1922, p. 53) that dimensional analysis may not be applicable to the results of most kinds of biological measurements, since in many cases these cannot be described in complete equations without the use of as many dimensional constants as there occur physical variables. This state of affairs arises from the fact that the mechanism whereby a given physical variable influences the biological manifestation considered is not only unknown, but is indeed itself the objective of inquiry. When two kinds of secondary measures of the same performance property are to be correlated, however, this restriction need not enter.

This will depend on the wisdom and understanding exercised in the choice of variables, but in a given instance can be subjected to direct experimental control and test of the result. Such a test is given in the present

case by the determination of several properties of our dimensionless exponent Z,—its demonstrated invariance under certain sets of conditions, and its mode of alteration under other circumstances. There is no valid reason for the occurrence of *properties* found if Z is the outcome of statistical accident. The elementary logic of this situation is of precisely the same kind as that involved in the proof which has been given of the number of excitation elements theory in responses to visual flicker, and of the invariance of certain properties of this quantity (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *a*, *b*, *c;* 1938–39; Crozier, 1939.)

Unquestionably considerations of the same type apply to a number of really analogous situations in physics. Their generality from a dynamical standpoint merits brief discussion. For phenomena of sensory discrimination other than visual, homogeneous data on somaesthetic pressure (Holway and Crozier, 1937*b*) show that ΔP, for P constant, is proportional to $A^{1.1}$. A specific biological example among phenomena of a different order is not out of place. Consider the problem of measuring the reproductive performance of mice. Unquestionably the amount of mouse substance produced at a birth is influenced by many factors. Some of these can be ruled out by dealing with first litters borne by mothers of a genetically uniform strain at the same age. The productivity in these litters can be measured in two ways: (1) the number N of young in a litter, (2) the total mass W of the litter. The problem which gives the analogy with the subject of the present paper is that of determining the relation between W and N. Precisely the same reasoning as already used asserts that we should find

$$W = kN\frac{dW}{dN}, \tag{7}$$

and that k should be independent of W, N, size, and speed of growth. This says that $W = W_1N^k$, where W_1 = the weight of a litter of 1. It is found empirically that log W is a rectilinear function of log N, with a fractional exponent, and that k is the same for various kinds of mice and for various other kinds of mammals (Crozier and Enzmann, 1935–36), and k is thus quite independent of the growth rate, size or weight of litter, weight of a litter of 1, or species. The fact that W and N are different quantitative measures of the productive performance, on different kinds of scales, does not interfere with the prediction of the form of their interrelationship. Precisely similar reasoning, involving the use of *formal* equation (7), has been used to predict successfully the relationship between *variations* of performance of visual reactions, when excitability is measured along each of two coordinate axes (Crozier, 1935; Crozier, Wolf, and Zerrahn-Wolf, 1936–37*a*, *b*; Crozier and Holway, 1938), so that the illustration does not by any means stand alone.

For nonliving physical systems two instances may be conveniently cited for comparison. (1) The maximum (saturation) current (i) in a thermionic tube is determined by the space charge due to the electrons between the electrodes. The charge can be measured indirectly by the current; but it is due to, and also measurable by, the saturating applied voltage V. Consequently we should find the relationship between pairs of values of i, V, to be of the form

$$\log i = n \log V + C.$$

Langmuir's law shows it to be

$$i = kV^{3/2}$$

(Langmuir, 1913). (2) The internal energy of a black body radiator may be measured by observing its temperature (Kelvin); this gives the mean energy of the molecules in the radiator. This energy also determines the energy density in the radiator, and thus the energy density of the emitted radiation with which it is in equilibrium. Hence if T be the temperature and R the external radiation density, we must expect to find R and T interrelated in the form

$$\log R = k \log T + C.$$

It is well known that Stefan's law shows $R = aT^4$.

A further biological instance is not without point. The "hunger drive" of a rat is objectively expressed in the frequency with which (under certain controlled conditions) it devours pellets of food; this "drive" decreases with the number N of equivalent pellets it consumes in series, and is therefore a function of $1/N$; it is also a declining function of the time elapsed during consumption of the N particles (Skinner, 1931 *a*, *b*). Consequently we can write the form of the expected relation between N and t, since $1/N$ and $1/t$ at a given point in an eating series each measures in a different way and on a different scale the magnitude of the "eating potential." Without assumptions of any kind as to the nature of the drive mechanism, but on purely dimensional grounds, as in the cases already discussed, we can say that

$$k\,N = t\,dN/dt, \text{ if } k \text{ is a constant,}$$

or $N^k = Ct$.

This is the relationship found (Skinner, 1931). It says that the dimensional constant C is equal to N^k after the initial unit of elapsed time. It clearly would be incorrect to say that since, in the experimental arrangement used one and only one ($N = 1$) piece can be eaten at a time, we should subtract 1 from N in the equation, yet this is the homologue of the proposed subtraction of n_t in Wald's (1937–38) formula.

VI

In equations (1, 4, and 5) the value of the exponent Z is independent of the units of area and of intensity, but (as we have seen, section IV) it is not independent of the manner in which area of retinal image is increased. This means that it cannot be independent of the form of the image (or of its regional location). Thus for observer A. H. H. there are available tests with circular areas (right eye) foveally fixated at the center (Holway and Hurvich, 1938). These data give $Z = 0.402$, for $\log_{10}A$, to be compared with 0.267 for rectangular areas (section IV) with the same amount of scatter in ΔI_m as in the upper plot of Fig. 5.

When, however, we employ a very narrow band of light, with one end on the fovea, approximating a radius of the centrally fixated circular field, the relation of ΔI_0 to A becomes more complex (Table III and Fig. 6).

Roughly speaking, within the fovea (radius *ca.* 1°), Z has a high value approximating 1 (*cf.* also Abney, 1897). For wider areas the slope (Z) abruptly declines to about half. For the three observers we note in Fig. 6

TABLE III

Visual intensity thresholds (ΔI_0, photons) as a function of area increasing in steps of $\times 2$; white light; exposure time 0.04 second angular height of test patch 2°; each entry is the mean of five measurements; $\sigma_{\Delta I}$ is the root-mean-square deviation of one observation; one edge of the test patch was centrally fixated at the center of the fovea, increasing areas being spread out horizontally on the temporal aspect of the retina. Data on three observers (left eye, A.C.S.H., W.J.C.; right eye, A.H.H.). See Fig. 6.

Width, *degrees*	Obs.: A. H. H.		A. C. S. H.		W. J. C.	
	$\log \Delta I_0$	$\log \sigma_{\Delta I}$	$\log \Delta I_0$	$\log \sigma_{\Delta I}$	$\log \Delta I_0$	$\log \sigma_{\Delta I}$
0.2	$\bar{3}.757$	$\bar{4}.537$	$\bar{2}.214$	$\bar{3}.126$	$\bar{3}.968$	$\bar{4}.891$
	$\bar{3}.888$	$\bar{4}.797$	$\bar{2}.183$	$\bar{3}.135$	$\bar{3}.892$	$\bar{4}.528$
	$\bar{3}.781$	$\bar{4}.537$	$\bar{2}.201$	$\bar{3}.147$	$\bar{3}.915$	$\bar{4}.579$
	$\bar{3}.834$	$\bar{4}.541$	$\bar{2}.199$	$\bar{3}.119$	$\bar{3}.934$	$\bar{4}.941$
0.4	$\bar{3}.451$	$\bar{4}.699$	$\bar{3}.873$	$\bar{4}.704$	$\bar{3}.685$	$\bar{4}.382$
	$\bar{3}.584$	$\bar{4}.817$	$\bar{3}.814$	$\bar{4}.463$	$\bar{3}.654$	$\bar{4}.151$
	$\bar{3}.599$	$\bar{4}.660$	$\bar{3}.927$	$\bar{4}.740$	$\bar{3}.685$	$\bar{4}.461$
	$\bar{3}.590$	$\bar{4}.292$	$\bar{3}.876$	$\bar{4}.798$	$\bar{3}.655$	$\bar{4}.374$
0.8	$\bar{3}.238$	$\bar{4}.662$	$\bar{3}.583$	$\bar{4}.363$	$\bar{3}.367$	$\bar{4}.232$
	$\bar{3}.410$	$\bar{4}.603$	$\bar{3}.608$	$\bar{4}.335$	$\bar{3}.362$	$\bar{4}.363$
	$\bar{3}.389$	$\bar{4}.370$	$\bar{3}.517$	$\bar{4}.241$	$\bar{3}.362$	$\bar{4}.787$
	$\bar{3}.230$	$\bar{4}.351$	$\bar{3}.668$	$\bar{4}.389$	$\bar{3}.373$	$\bar{4}.113$
1.6	$\bar{4}.929$	$\bar{5}.849$	$\bar{3}.453$	$\bar{4}.577$	$\bar{3}.161$	$\bar{4}.063$
	$\bar{3}.053$	$\bar{4}.096$	$\bar{3}.352$	$\bar{4}.247$	$\bar{3}.158$	$\bar{4}.136$
	$\bar{3}.100$	$\bar{4}.170$	$\bar{3}.419$	$\bar{4}.232$	$\bar{3}.137$	$\bar{5}.987$
	$\bar{3}.033$	$\bar{4}.065$	$\bar{3}.398$	$\bar{4}.595$	$\bar{3}.152$	$\bar{5}.916$
3.2	$\bar{4}.754$	$\bar{5}.730$	$\bar{3}.250$	$\bar{4}.0587$	$\bar{4}.981$	$\bar{4}.084$
	$\bar{4}.705$	$\bar{5}.875$	$\bar{3}.193$	$\bar{5}.803$	$\bar{3}.017$	$\bar{4}.173$
	$\bar{4}.717$	$\bar{5}.693$	$\bar{3}.204$	$\bar{4}.222$	$\bar{3}.065$	$\bar{4}.121$
	$\bar{4}.772$	$\bar{5}.668$	$\bar{3}.182$	$\bar{5}.946$	$\bar{4}.966$	$\bar{5}.707$
6.4	$\bar{4}.602$	$\bar{5}.583$	$\bar{3}.004$	$\bar{4}.010$	$\bar{4}.830$	$\bar{5}.804$
	$\bar{4}.623$	$\bar{5}.617$	$\bar{3}.089$	$\bar{5}.937$	$\bar{4}.851$	$\bar{5}.846$
	$\bar{4}.609$	$\bar{5}.554$	$\bar{3}.102$	$\bar{4}.069$	$\bar{4}.803$	$\bar{5}.753$
	$\bar{4}.651$	$\bar{5}.661$	$\bar{3}.018$	$\bar{5}.976$	$\bar{4}.828$	$\bar{5}.835$
9.6	$\bar{4}.483$	$\bar{5}.380$	$\bar{4}.908$	$\bar{5}.531$	$\bar{4}.774$	$\bar{5}.820$
	$\bar{4}.528$	$\bar{5}.486$	$\bar{4}.969$	$\bar{5}.961$	$\bar{4}.680$	$\bar{5}.4137$
	$\bar{4}.558$	$\bar{5}.423$	$\bar{4}.940$	$\bar{5}.740$	$\bar{4}.743$	$\bar{5}.626$
	$\bar{4}.598$	$\bar{5}.471$	$\bar{4}.942$	$\bar{5}.830$	$\bar{4}.666$	$\bar{5}.586$

certain individual differences, which may be directly correlated with already known peculiarities of their individual local excitability thresholds in the same horizontal retinal meridian (Crozier and Holway, 1938–39 *b*). In the

first place the general order of the excitabilities (A.H.H. > W.J.C. > A.C.S.H.) is the same. More interesting is the fact that the ΔI_0 distance

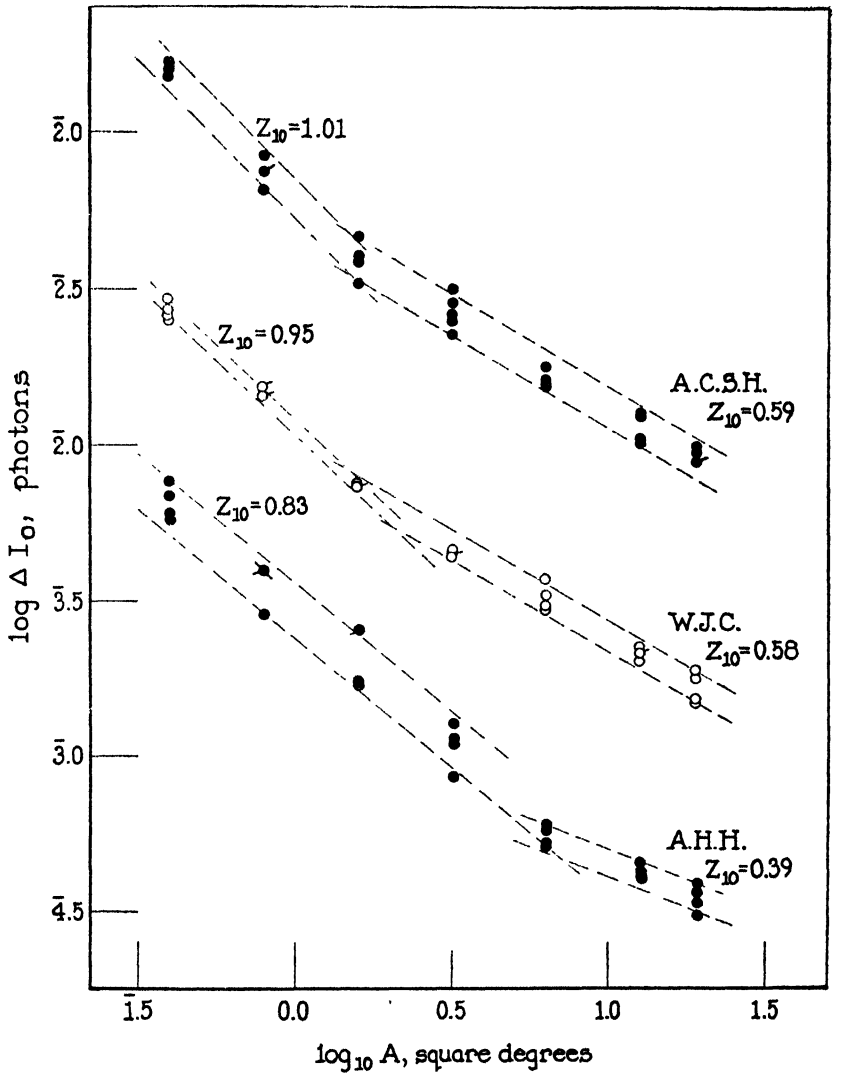

FIG. 6. Dependence of ΔI_m upon A when the image is a narrow bar of increasing length; see text; data in Table III.

between excitabilities at the fovea and in the extra-foveal region is much the greatest for A.H.H., least for A.C.S.H., and intermediate for W.J.C. (*cf.* Crozier and Holway, 1938-39 *b*, Fig. 4 on p. 356). In correlation with

this we find (Fig. 6) that the lower slope for A.H.H. is least, and that for A.H.H. the higher (foveal) slope is continued to a much more extensive area, the order of appearance of the "break" being A.C.S.H. < W.J.C. < A.H.H. This clearly signifies that the intrinsic excitability of each included region contributes to the determination of ΔI_0, but that it is the magnitude

TABLE IV

Visual intensity thresholds ΔI_0, (photons) as a function of area increasing in steps of $\times 2$; white light, exposure time 0.04 second; angular height of test patch 2° at the retina; each entry is the mean of five measurements; $\sigma_{\Delta I}$ is the root-mean-square of one observation; one edge of the test patch was centrally fixated at the center of the fovea; increasing areas A_1, A_2, A_3, A_4 were spread out horizontally on the temporal aspect of the retina; see text. Data on three observers (left eye, W.J.C., A.C.S.H.; right eye, A.H.H.).

Obs.: W. J. C.				A. C. S. H.				A. H. H.			
$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$	$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$	$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$	$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$	$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$	$\log \Delta I_0$	$\log \sigma_{\Delta I_0}$
$A_1 = 1.2°$		$(A_2 - A_1) = 1.2°$		$A_1 = 1.2°$		$(A_2 - A_1) = 1.2°$		$A_1 = 1.2°$		$(A_2 - A_1) = 1.2°$	
$\bar{3}.344$	$\bar{4}.543$	$\bar{3}.295$	$\bar{4}.252$	$\bar{3}.485$	$\bar{4}.495$	$\bar{3}.393$	$\bar{4}.415$	$\bar{3}.017$	$\bar{4}.183$	$\bar{4}.899$	$\bar{5}.924$
$\bar{3}.288$	$\bar{4}.525$	$\bar{3}.308$	$\bar{4}.463$	$\bar{3}.492$	$\bar{4}.503$	$\bar{3}.361$	$\bar{4}.451$	$\bar{3}.096$	$\bar{4}.002$	$\bar{4}.876$	$\bar{5}.884$
$\bar{3}.243$	$\bar{4}.428$	$\bar{3}.220$	$\bar{4}.689$	$\bar{3}.470$	$\bar{4}.688$	$\bar{3}.396$	$\bar{4}.300$	$\bar{3}.074$	$\bar{4}.208$	$\bar{4}.955$	$\bar{5}.987$
$\bar{3}.243$	$\bar{4}.613$	$\bar{3}.233$	$\bar{4}.501$	$\bar{3}.459$	$\bar{4}.752$	$\bar{3}.348$	$\bar{4}.702$	$\bar{3}.112$	$\bar{4}.193$	$\bar{4}.899$	$\bar{4}.127$
$A_2 = 2.4°$		$(A_3 - A_2) = 2.4°$		$A_2 = 2.4°$		$(A_3 - A_2) = 2.4°$		$A_2 = 2.4°$		$(A_3 - A_2) = 2.4°$	
$\bar{3}.179$	$\bar{4}.344$	$\bar{3}.173$	$\bar{4}.402$	$\bar{3}.250$	$\bar{4}.397$	$\bar{3}.090$	$\bar{4}.091$	$\bar{4}.803$	$\bar{4}.143$	$\bar{4}.807$	$\bar{4}.001$
$\bar{3}.117$	$\bar{4}.266$	$\bar{3}.185$	$\bar{4}.199$	$\bar{3}.262$	$\bar{4}.353$	$\bar{3}.146$	$\bar{4}.256$	$\bar{4}.870$	$\bar{4}.121$	$\bar{4}.723$	$\bar{5}.983$
$\bar{3}.193$	$\bar{4}.378$	$\bar{3}.152$	$\bar{4}.616$	$\bar{3}.250$	$\bar{4}.411$	$\bar{3}.041$	$\bar{4}.099$	$\bar{4}.856$	$\bar{4}.067$	$\bar{4}.732$	$\bar{5}.939$
$\bar{3}.145$	$\bar{4}.318$	$\bar{3}.158$	$\bar{4}.031$	$\bar{3}.239$	$\bar{4}.295$	$\bar{3}.013$	$\bar{4}.262$	$\bar{4}.831$	$\bar{5}.931$	$\bar{4}.743$	$\bar{5}.778$
$A_3 = 4.8°$		$(A_4 - A_3) = 4.8°$		$A_3 = 4.8°$		$(A_4 - A_3) = 4.8°$		$A_3 = 4.8°$		$(A_4 - A_3) = 4.8°$	
$\bar{3}.029$	$\bar{5}.980$	$\bar{4}.918$	$\bar{4}.001$	$\bar{4}.952$	$\bar{5}.986$	$\bar{4}.958$	$\bar{4}.044$	$\bar{4}.629$	$\bar{4}.004$	$\bar{4}.611$	$\bar{5}.624$
$\bar{3}.061$	$\bar{4}.332$	$\bar{4}.975$	$\bar{4}.503$	$\bar{4}.949$	$\bar{5}.999$	$\bar{4}.896$	$\bar{4}.006$	$\bar{4}.664$	$\bar{5}.580$	$\bar{4}.622$	$\bar{5}.714$
$\bar{3}.021$	$\bar{4}.996$	$\bar{4}.921$	$\bar{4}.286$	$\bar{4}.982$	$\bar{5}.991$	$\bar{4}.947$	$\bar{4}.102$	$\bar{4}.652$	$\bar{5}.579$	$\bar{4}.536$	$\bar{5}.865$
$\bar{3}.041$	$\bar{4}.253$	$\bar{4}.884$	$\bar{5}.689$	$\bar{4}.903$	$\bar{4}.057$	$\bar{4}.902$	$\bar{4}.082$	$\bar{4}.702$	$\bar{5}.900$	$\bar{4}.555$	$\bar{5}.935$
$A_4 = 9.6°$				$A_4 = 9.6°$				$A_4 = 9.6°$			
$\bar{4}.844$	$\bar{4}.232$			$\bar{4}.832$	$\bar{5}.907$			$\bar{4}.619$	$\bar{5}.844$		
$\bar{4}.827$	$\bar{5}.696$			$\bar{4}.817$	$\bar{5}.757$			$\bar{4}.572$	$\bar{5}.724$		
$\bar{4}.831$	$\bar{4}.302$			$\bar{4}.846$	$\bar{4}.087$			$\bar{4}.592$	$\bar{5}.776$		
$\bar{4}.839$	$\bar{5}.860$			$\bar{4}.821$	$\bar{4}.124$			$\bar{4}.600$	$\bar{5}.801$		

of $d(1/\Delta I_0)/dA$, *i.e.* the rate of addition of excitability units as A is increased, which is a governing factor.

The relationship is not simply one of addition, however. It is not our present purpose to discuss the phenomena and theory of "retinal summation," but we are required to demonstrate that there is a sense in which a given stimulated retinal area behaves as a unit, and in which its contribu-

tion to the observed excitability is determined by the concurrent excitation of spatially contiguous regions. An experiment giving such a demonstration is summarized in Table IV. As in the experiment of Table III, one 2° edge of the test patch was centered at the fovea; ΔI_0 was first determined for a bar 1.2° wide (A_1); then for one 2.4° wide (A_2); then for the outer half of A_2, *i.e.* for $A_2 - A_1$; and so on, as indicated in Table IV. It is apparent that under these conditions for all three observers the threshold for a given rectangular area is *less* than for either the near-foveal half or the outer half of the area alone, although for a given area the threshold is higher for the near-foveal half than for the outer half. The plot of log ΔI_0 *vs.* log A for these data shows that Z agrees very well with the values for each observer (Fig. 6) gotten with the rectangular patch 12.8° high (Table III). Hence we have direct support for the view that it is retinal *area* rather than visual angle which is the proper independent variable. It is also apparent in the data of Table IV that the slopes for log ΔI_0 *vs.* A are practically the same for A_1, A_2, A_3, A_4, as for $A_2 - A_1$, $A_3 - A_2$, *etc;* hence these strips behave as if essentially "homogeneous" so far as the resultant relative rate of increase of excitation is concerned.

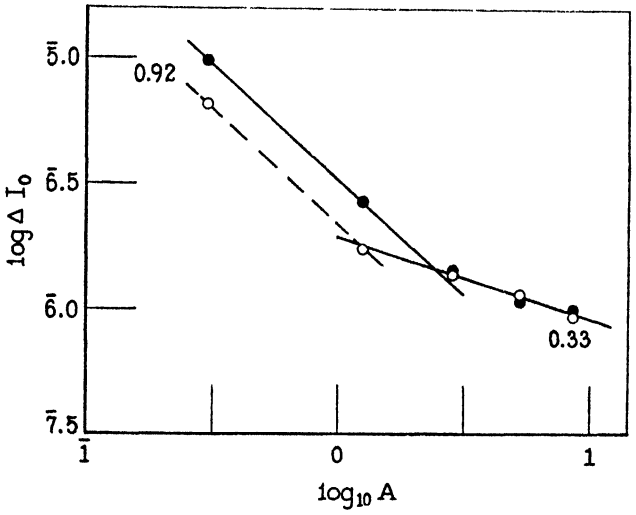

FIG. 6 *bis*. Data from Wald (1937–38, his Table II, p. 272): threshold ΔI_0 (millilamberts, no artificial pupil, *ca.* 1 second exposure) as a function of circular area (A, arbitrary units proportional to square centimeters) for two regions respectively centered 15° above the fovea (open circlets) and 25° above (solid circlets). Within the error of such measurements the slopes, $Z = 0.92$ and 0.33 as drawn, agree quantitatively with those in our Fig. 6—see particularly the plot for A.H.H.; it is to be noted also that the break in the curve comes in precisely the same zone of absolute area of retinal image.

We desire to avoid any specific discussion of the relationship between the two slopes appearing in Fig. 6 and the properties traditionally assumed for cones and rods respectively. These slopes are of the order of 0.93 and

0.52. (For the data in Fig. 3, Table I, the value of Z is 0.267, but it is to be kept in mind that Z must in all likelihood be a decreasing function of exposure time—*ca.* 30 seconds in the latter case, and 0.40 second in the former.) For small regions 15° and 25° from the fovea, Wald's primary data (1937–38) show (Fig. 6 *bis*) exactly the same sort of break as we have demonstrated in Fig. 6. Piéron (1929) cites foveal measurements which "break" at about the same area ($\log_{10} A = 0.7 \pm$). It is impossible to hold that the high slope is due to the functioning of retinal cones, since at 15° away from the fovea the proportion of cones to rods is about 1:25, while at 25° away, with a smaller number of rods per unit area, the proportion is only slightly less; the assumption that rod thresholds are intrinsically much lower than cone thresholds cannot be used to explain the facts shown in Figs. 6 and 6 *bis*. Obviously, if the low-slope segment, at the larger areas, is due to the threshold activation of rods, there is no reason for supposing that with the smallest areas the decreasing number of high-threshold cones would alone become effective.

VII

Monocular Observations, Colored Light

Monocular determinations of ΔI_m were made for red, green, and blue lights. The method of observation was the same as with white light. The filters and the calibration of intensities are discussed in section II. The data are summarized in Table V.

In Fig. 7 these measurements are plotted in terms of equation (5). The fitted lines give mean $Z = 0.239$; when A is taken logarithmically to base 2, $Z = 0.080$. A twofold increase of area occasions a reduction of ΔI_m by a factor of 1.202. The mean values of Z ($= 0.239$) are less than that obtained with white light ($Z = 0.267$). The reality of the lower slope is supported by the similar difference obtained in the series of binocular measurements (section VIII). The differences between the slopes for the three kinds of colored lights are not certainly significant (Fig. 8), but are reasonably consistent with certain expectations. In Fig. 8 the data for each color are brought together for comparison: in each set the ΔI_m's are multiplied by a constant, as in Fig. 5 for white light.

The discussion in section VI makes it clear, we believe, that if the absolute number of elements open to excitation is less, as result of a given method of presentation, then the slope of the log ΔI *vs.* log A plot must be expected to be less. Specifically, since in energy terms the thresholds for "mono-

chromatic" light are higher than for white, fewer elements of effect are open to arousal; consequently, with a given area, a given percentage increase of

TABLE V

Colored lights: homogeneous results for ΔI and A, A.H.H., right eye. Intensity and exposure time (0.04 second) were parameters. I_1 is the standard intensity, in photons. A, in degrees, is the angular width of the light image on the retina; the height of the image was constant = 20.8°. Each ΔI entry is an average of five measurements; $\sigma_{\Delta I}$ (log, in parentheses) is the root-mean-square variation of a single observation. See Figs. 7 and 8.

log I_1, *photons*	A = 0.4°	0.8°	1.6°	3.2°	6.4°	12.8°
			λ = 465			
$\bar{1}$.334	$\bar{2}$.093	$\bar{3}$.964	$\bar{3}$.901	$\bar{3}$.802	$\bar{3}$.794	$\bar{3}$.628
	($\bar{3}$.082)	($\bar{4}$.943)	($\bar{4}$.916)	($\bar{4}$.827)	($\bar{4}$.698)	($\bar{4}$.617)
0.334	2.987	$\bar{2}$.793	$\bar{2}$.841	$\bar{2}$.650	$\bar{2}$.629	$\bar{2}$.601
	($\bar{2}$.914)	($\bar{3}$ 816)	($\bar{3}$.873)	($\bar{3}$.779)	($\bar{3}$.593)	($\bar{3}$.615)
1.334	$\bar{1}$.923	$\bar{1}$.688	$\bar{1}$.811	$\bar{1}$.680	$\bar{1}$.627	$\bar{1}$.425
	($\bar{1}$.000)	($\bar{2}$.653)	($\bar{2}$ 804)	($\bar{2}$.697)	($\bar{2}$.656)	($\bar{2}$.601)
2.334	0.802	0.703	0.653	0.528	0.521	0.477
	(1.914)	($\bar{1}$.896)	($\bar{1}$.633)	($\bar{1}$.577)	($\bar{1}$.606)	($\bar{1}$.500)
3.334	1.610	1.469	1.583	1.399	1.370	1.294
	(0.573)	(0.458)	(0.600)	(0.404)	(0 380)	(0.305)
			λ = 525			
$\bar{1}$.2	$\bar{2}$.614	$\bar{2}$.563	$\bar{2}$.472	$\bar{2}$.419	$\bar{2}$.343	$\bar{2}$.310
	($\bar{3}$.714)	($\bar{3}$.606)	($\bar{3}$.513)	($\bar{3}$.558)	($\bar{3}$.316)	($\bar{3}$.354)
0.2	$\bar{1}$.501	$\bar{1}$.492	$\bar{1}$.387	$\bar{1}$ 369	$\bar{1}$.216	$\bar{1}$.216
	($\bar{2}$.497)	($\bar{2}$.515)	($\bar{2}$.463)	($\bar{2}$.494)	($\bar{2}$ 365)	($\bar{2}$.227)
1.2	0.248	0.216	0.076	0.032	0.032	$\bar{1}$.897
	($\bar{1}$.290)	($\bar{1}$.307)	($\bar{1}$.004)	($\bar{1}$.006)	($\bar{2}$.979)	($\bar{2}$.914)
2.2	1.230	1.160	1.056	1 032	0.977	0.915
	(0.293)	0.102	0.125	$\bar{1}$.993	0.043	$\bar{1}$.976
			λ = 680			
$\bar{1}$.49	$\bar{1}$.397	$\bar{1}$.285	$\bar{1}$.246	$\bar{1}$ 125	$\bar{1}$.079	$\bar{2}$.958
	($\bar{2}$.388)	($\bar{2}$.209)	($\bar{2}$.271)	($\bar{2}$.135)	($\bar{2}$.092)	($\bar{3}$.896)
0.49	0.250	0.224	0.128	0 053	$\bar{1}$.996	$\bar{1}$.892
	($\bar{1}$.138)	($\bar{1}$.243)	($\bar{1}$.207)	($\bar{1}$.119)	($\bar{2}$.943)	($\bar{2}$.939)
1.49	0.848	0.716	0.699	0.634	0.602	0.544
	($\bar{1}$.794)	($\bar{1}$.806)	($\bar{1}$.703)	($\bar{1}$.619)	($\bar{1}$.654)	($\bar{1}$.582)
2.49	1.799	1.763	1.634	1.544	1.477	1 372
	(0.800)	(0.772)	(0.705)	(0.593)	(0.484)	(0.405)

area, by the same method, should produce a smaller relative decrease of ΔI (for a given I_1). This is what the data of Table V show.

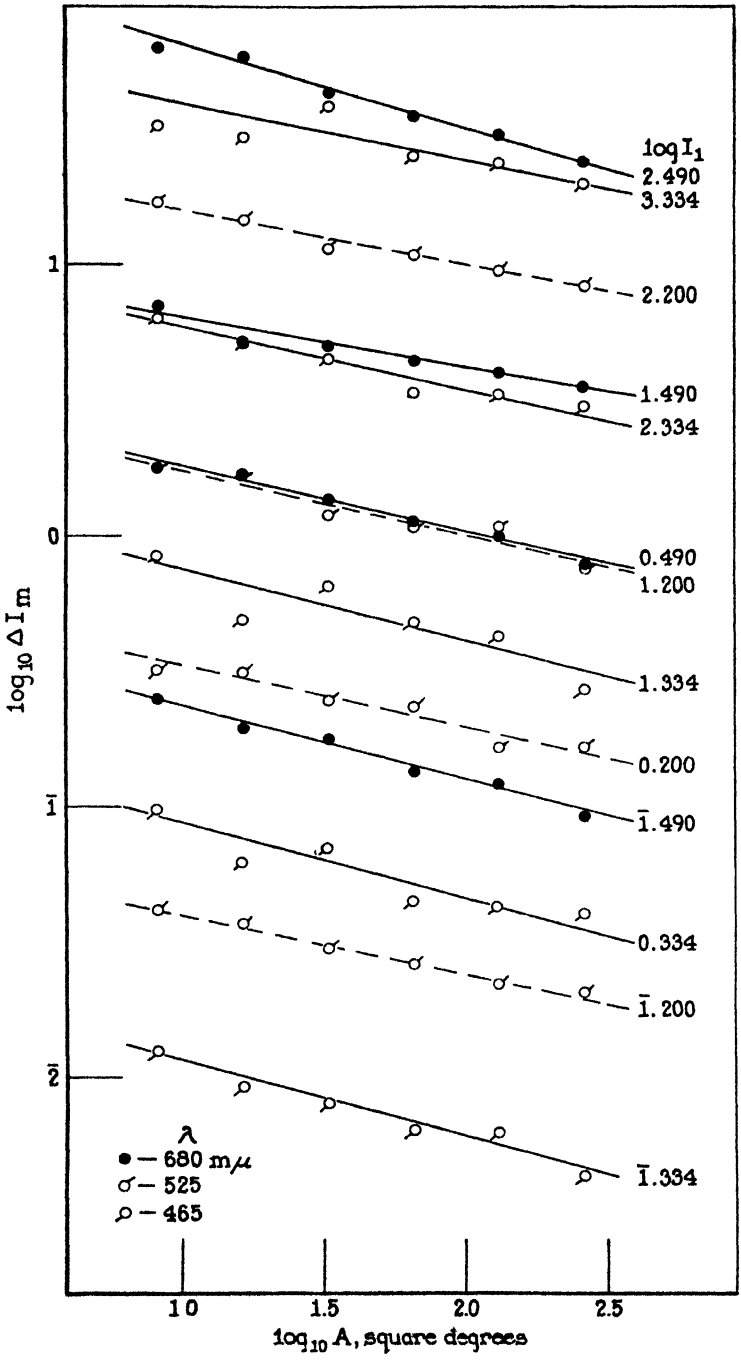

FIG. 7. Monocular excitation, as in Fig. 3, but with I_1 and λ as parameters (Table V).

VIII

Binocular Measurements

The form of the function relating ΔI_m to area for binocular excitation was determined by means of homogeneous data secured under conditions essentially identical with those used in the monocular procedure. The same apparatus, filters, wedges, and observers were used. Matched oculars, instead of a single ocular, were placed in the discriminometer head

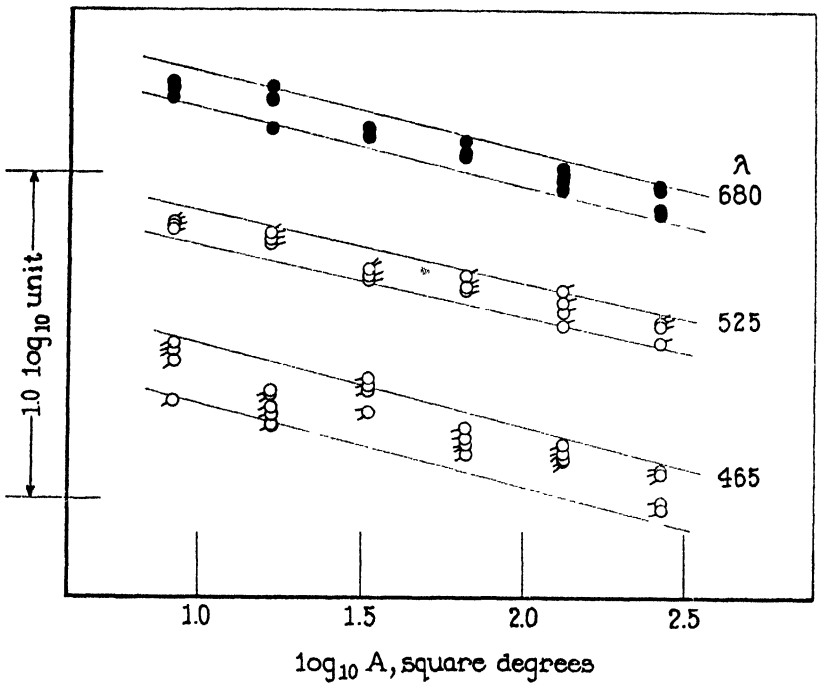

FIG. 8. The plots of Fig. 7 brought together for comparison of slopes (Z) for each of the three colors used; see text.

(section II). Measurements were taken only during times when the observer experienced a perfectly fused, unitary visual impression.

A certain amount of confusion still prevails with respect to the comparison of monocular and binocular thresholds. The pupillary reaction to the effects of photic excitation is consensual. For the commonplace situation, the size of the pupil in either eye is smaller when both eyes are stimulated by a given intensity than when one eye alone is stimulated, as is well known (*cf.* Rea, 1938). This fact clearly requires the use of effective artificial

pupils in experiments designed to test the absolute magnitudes of ΔI for binocular as contrasted with monocular discrimination. In addition, the importance of placing *equivalent* light images at corresponding points on the two retinae necessitates providing conditions which eliminate differences of form, size, and intensity distribution in the images used for both eyes as well as for one eye. This is done by assuring absence of changes in effective

TABLE VI

White light.—homogeneous results for *binocular* ΔI and A. I_1 is standard intensity, in photons. A', in degrees, is the angular width of the light image on the retina; the height of the image was constant, = 20.8°. Each ΔI entry is an average of five measurements; $\sigma_{\Delta I}$ is the root-mean-square variation of a single measurement. Observer: A.H.H. See Figs. 9 and 10.

Colored lights.—homogeneous results for *binocular* ΔI and A'. Intensity and exposure-time were parameters. I_1 is the standard intensity, in photons. A, in degrees, is the angular width of the light image on the retina; the height of the image was constant = 20.8°. Each ΔI entry is an average of five measurements; $\sigma_{\Delta I}$ is the root-mean-square variation of a single observation. See Figs. 9 and 10.

log I, *photons*	A' = 0.4°	0.8°	1.6°	3.2°	6.4°	12.8°
0.0	$\bar{1}$.217	$\bar{1}$.150	$\bar{1}$.021	$\bar{2}$.984	$\bar{2}$.861	$\bar{2}$.777
	($\bar{2}$.039)	($\bar{2}$.215)	($\bar{2}$.296)	($\bar{2}$.051)	($\bar{2}$.120)	($\bar{3}$.753)
1.0	0.153	0.080	$\bar{1}$.964	$\bar{1}$.885	$\bar{1}$.846	$\bar{1}$.730
	($\bar{1}$.217)	($\bar{1}$.193)	($\bar{1}$.045)	($\bar{1}$.126)	($\bar{2}$.803)	($\bar{2}$.821)
2.0	1.041	0.997	0.906	0.785	0.703	0.644
	(0.050)	(0.107)	(0.089)	($\bar{1}$.892)	($\bar{1}$.757)	($\bar{1}$.684)
3.0	2.050	1.903	1.941	1.867	1.680	1.652
	(1.132)	(1.082)	(1.112)	(1 002)	(0.679)	(0.829)
1.334	$\bar{1}$.783	$\bar{1}$.692	$\bar{1}$.625	$\bar{1}$.551	$\bar{1}$.443	$\bar{1}$.300
λ = 465	($\bar{2}$.816)	($\bar{2}$.753)	($\bar{2}$.656)	($\bar{2}$.701)	($\bar{2}$.409)	($\bar{2}$.416)
2.200	1.283	1.210	1.129	1.079	1.044	0.930
λ = 525	(0.288)	(0.312)	(0.065)	(0.207)	($\bar{1}$.993)	($\bar{1}$.942)
1.490	0.813	0.726	0.683	0.650	0.555	0.512
λ = 680	($\bar{1}$.927)	($\bar{1}$.735)	($\bar{1}$.702)	($\bar{1}$.795)	($\bar{1}$.701)	($\bar{1}$.553)

pupil aperture and by maintaining completely relaxed accommodation (Crozier and Holway, 1938–39 *a*, *b*). The former is given by the ocular eye ring in our apparatus (constant and less than 1.8 mm. diameter in the plane of the pupil of the observer's eye), the latter by the presence of a suitable small fixation point sharply defined at the retina only under completely relaxed accommodation (Crozier and Holway, 1938–39 *a*). It appears to us that only under conditions such as these can a real answer be

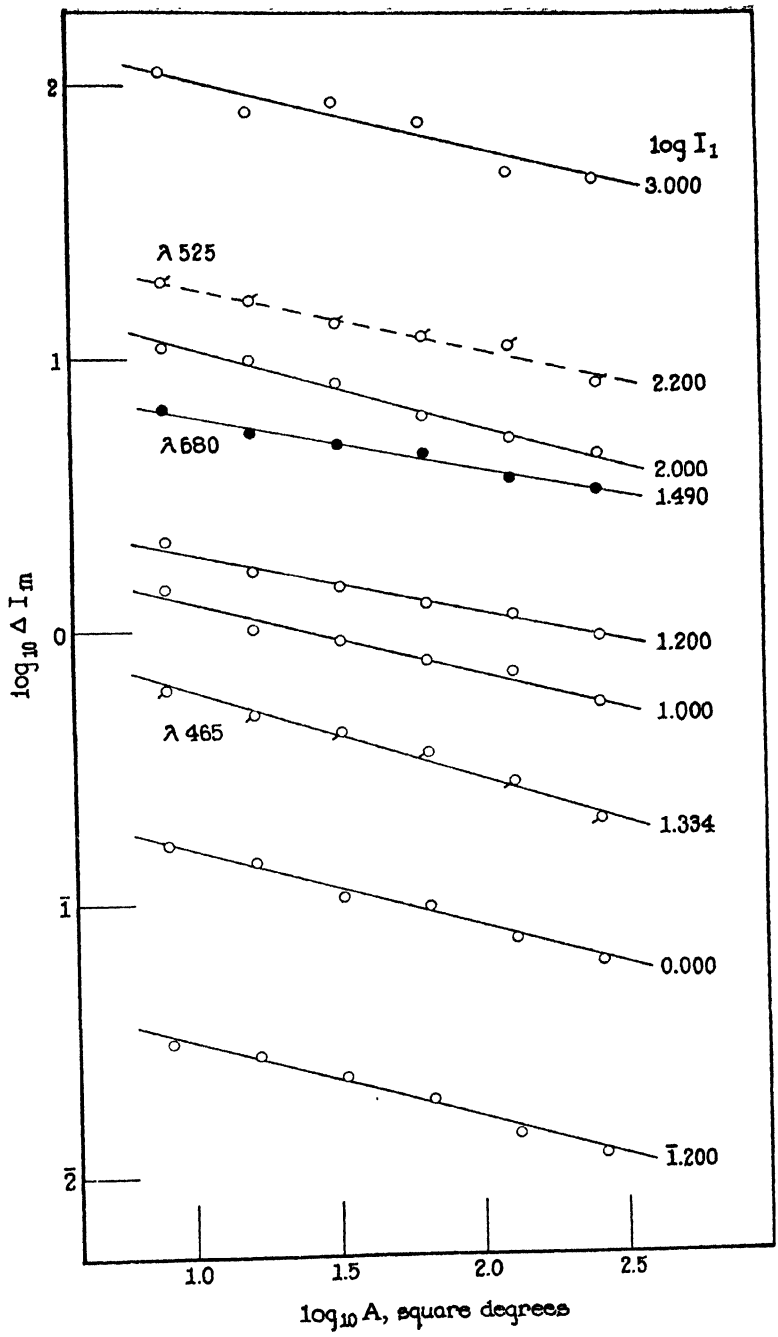

FIG. 9. Dependence of ΔI_m on A for simultaneous excitation of *both* eyes (*i.e.*, "binocular" presentation), at several levels of I_1 and for three colored lights. Table VI.

obtained to the question as to whether a valid difference exists between monocular and binocular thresholds for constant retinal light images.

When these conditions are effectively maintained, binocular absolute thresholds are found to be lower than for either eye separately (*cf.* Crozier and Holway, 1938–39 *b*). There seems to be no possibility of explaining this result through technical error of observation. Failure to maintain

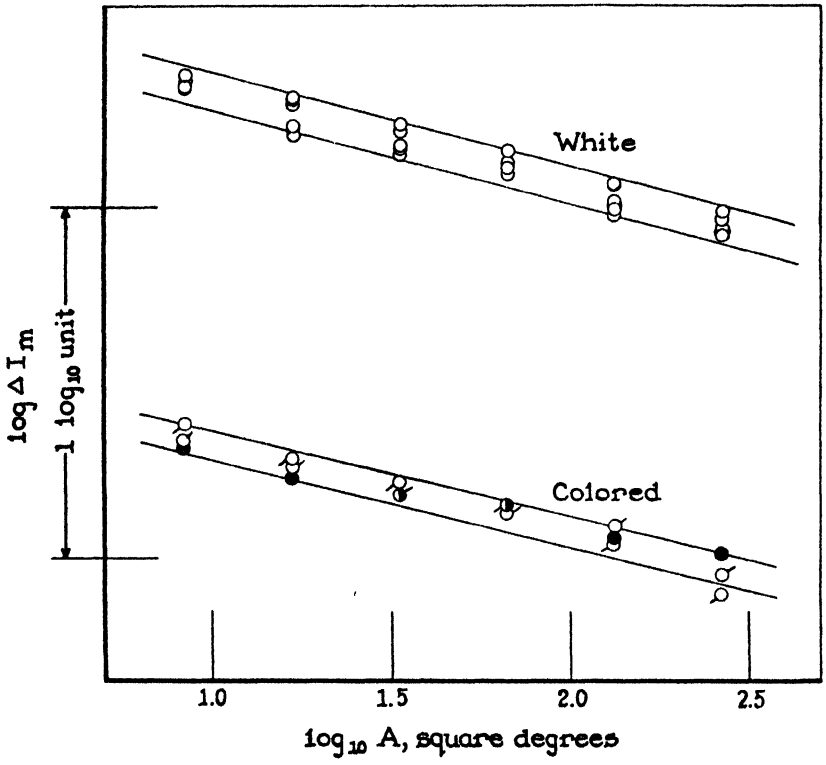

FIG. 10. Data of Fig. 9 brought together for comparison of slopes (Z), white light (above) and colored (below).

such conditions can easily account for data in which the binocular thresholds (absolute or differential) appear either as the average of those for each eye, or as approximately equal to those for the more excitable eye.

Data for white light are given in Table VI and in Fig. 9. In Table VI the visual angle A' (degrees) is the average of the equal angles subtended at each eye. I_1 is the standard intensity (photons). Each I_B entry is the mean of five measurements; $\sigma_{\Delta I_B}$ is the root-mean-square deviation. As with the monocular measurements (section III, *etc.*), both ΔI_B and $\sigma_{\Delta I_B}$

are decreasing functions of A for I_1 as parameter, and increasing functions of I_1 when A is constant. Equation (5) is adequate for these data (Fig. 9) as well as for the monocular observations (Figs. 4 and 5). The mean slope constant $Z = 0.268$ (Fig. 10), and is not significantly higher than the 0.267 for the monocular white data.

Table VI also contains corresponding data for three colored lights (Figs. 9 and 10). The mean value of Z (0.243) is a little greater than that for monocular presentation (sections III and VII), but the difference is probably not significant. For both monocular and binocular measurements Z is *smaller* with monochromatic light than with white. To a very close approximation $\log \Delta I_m$ decreases with respect to increase of $\log A$ at the same rate for monocular and for binocular excitation in each case. If binocular presentation of a given area to each eye actually "doubles" the number of affected elements, then a given increase of area will produce about the proportionate increase in potentially excitable elements in case of two eyes as when one is used, and our Z should not change appreciably.

It will be noticed that in this formulation Z is held to be essentially independent of changes produced in ΔI by means other than alteration of area, except in so far as Z is dependent on λ. The monocular and binocular values of ΔI thus far considered differ at fixed I_1 by a small but definite amount. This has been tested by plotting (from Figs. 3, 7, and 9) the values of $\log \Delta I_m$ at $A = 10$ units against the corresponding values of $\log I_1$. These data, however, are not most suitable for the determination of intrinsic differences between monocular and binocular excitabilities. Series of determinations were therefore made by observing ΔI (single measurement) with one eye, then with the other, and then with both, until five sets of readings were secured at a given A and I_1 (*cf.* Crozier and Holway, 1938–39 *b*).

Homogeneous data secured in this way are given in Table VII. Two of these series, $\log I_1 = \bar{1}.20$ and 1.20, were obtained by the method used in Table I, namely by symmetrical enlargement of the area on either side of the fovea; at another, intermediate intensity ($\log I_1 = 0.80$) the method of Table II was employed, area being enlarged by geometrically progressive steps toward the fovea from a fixed margin 12.8° on the temporal side (*cf.* section IV). It is to be noted that, as in the case of "absolute" threshold measurements with small areas at various retinal positions (Crozier and Holway, 1938–39 *b*), small differences are apparent in the readings with the two eyes. For the observer used in Table VII we have already found these differences to be comparatively minute. This is for certain purposes a distinct advantage. In the series under present consideration the differ-

TABLE VII

Comparable results for binocular and monocular ΔI. Angular breadth, A', intensity, I_1, wave-length composition, and exposure time (0.04 second) are parameters. For the upper and the lower curves (log I_1 = $\bar{1}.20$ and 1.20), all images were *centrally* fixated. A', in degrees, is the angular width of the image on the retina (height, 20.8°). I_1 is the standard intensity. For log I_1 = 0.80 (middle curve) the outer margin of the test patch was at 12.8° from the fovea (*see text*). Each ΔI_m value is an average of five measurements; $\sigma_{\Delta I}$ (in parentheses) is the root-mean-square variation of a single observation. ΔI_B is for binocular regard; ΔI_L, and ΔI_R are the values for the left and right eyes respectively. Observer, A.H.H. For discussion, see text. Fig. 11.

log I_1	log ΔI_L	log ΔI_R	log ΔI_B	
$\bar{1}.20$	$\bar{2}.641$	$\bar{2}.609$	$\bar{2}.490$	A' = 0.4°
	($\bar{3}.575$)	($\bar{3}.721$)	($\bar{3}.583$)	
	$\bar{2}.559$	$\bar{2}.500$	$\bar{2}.441$	A' = 0.8
	($\bar{3}.560$)	($\bar{3}.612$)	($\bar{3}.603$)	
	$\bar{2}.521$	$\bar{2}.480$	$\bar{2}.362$	A' = 1.6
	($\bar{3}.619$)	($\bar{3}.505$)	($\bar{3}.392$)	
	$\bar{2}.401$	$\bar{2}.396$	$\bar{2}.275$	A' = 3.2
	($\bar{3}.507$)	($\bar{3}.510$)	($\bar{3}.300$)	
	$\bar{2}.325$	$\bar{2}.316$	$\bar{2}.146$	A' = 6.4
	($\bar{3}.367$)	($\bar{3}.355$)	($\bar{3}.099$)	
	$\bar{2}.248$	$\bar{2}.219$	$\bar{2}.068$	A' = 12.8
	($\bar{3}.178$)	($\bar{3}.192$)	($\bar{3}.108$)	
1.20	0.482	0.511	0.329	A' = 0.4°
	($\bar{1}.376$)	($\bar{1}.635$)	($\bar{1}.442$)	
	0.449	0.365	0.210	A' = 0.8
	($\bar{1}.516$)	($\bar{1}.252$)	($\bar{1}.307$)	
	0.257	0.271	0.159	A' = 1.6
	($\bar{1}.379$)	($\bar{1}.302$)	($\bar{1}.162$)	
	0.160	0.209	0.097	A' = 3.2
	($\bar{1}.103$)	($\bar{1}.294$)	($\bar{1}.085$)	
	0.281	0.220	0.057	A' = 6.4
	($\bar{1}.392$)	($\bar{1}.163$)	($\bar{1}.066$)	
	0.112	0.100	$\bar{1}.973$	A' = 12.8
	($\bar{1}.241$)	($\bar{1}.092$)	($\bar{1}.041$)	
0.80	$\bar{1}.905$	$\bar{1}.867$	$\bar{1}.782$	A' = 0.4
	($\bar{2}.882$)	($\bar{2}.953$)	($\bar{2}.891$)	
	$\bar{1}.783$	$\bar{1}.783$	$\bar{1}.640$	A' = 0.8
	($\bar{2}.864$)	($\bar{2}.650$)	($\bar{2}.703$)	
	$\bar{1}.484$	$\bar{1}.499$	$\bar{1}.381$	A' = 1.6
	($\bar{2}.455$)	($\bar{2}.399$)	($\bar{2}.457$)	
	$\bar{1}.407$	$\bar{1}.366$	$\bar{1}.241$	A' = 3.2
	($\bar{2}.512$)	($\bar{2}.402$)	($\bar{2}.286$)	
	$\bar{1}.362$	$\bar{1}.279$	$\bar{1}.115$	A' = 6.4
	($\bar{2}.453$)	($\bar{2}.265$)	($\bar{2}.105$)	
	$\bar{1}.043$	$\bar{1}.075$	$\bar{2}.920$	A' = 12.8
	($\bar{2}.031$)	($\bar{2}.102$)	($\bar{1}.991$)	

ences between $\Delta \bar{I}_L$ and $\Delta \bar{I}_R$ are probably not significant. For each value of a_1, and for all values of A, $\Delta \bar{I}_B$ is seen to be smaller than ΔI_L or ΔI_R. Using equation (5) to describe these data (Fig. 11), the mean value of Z is

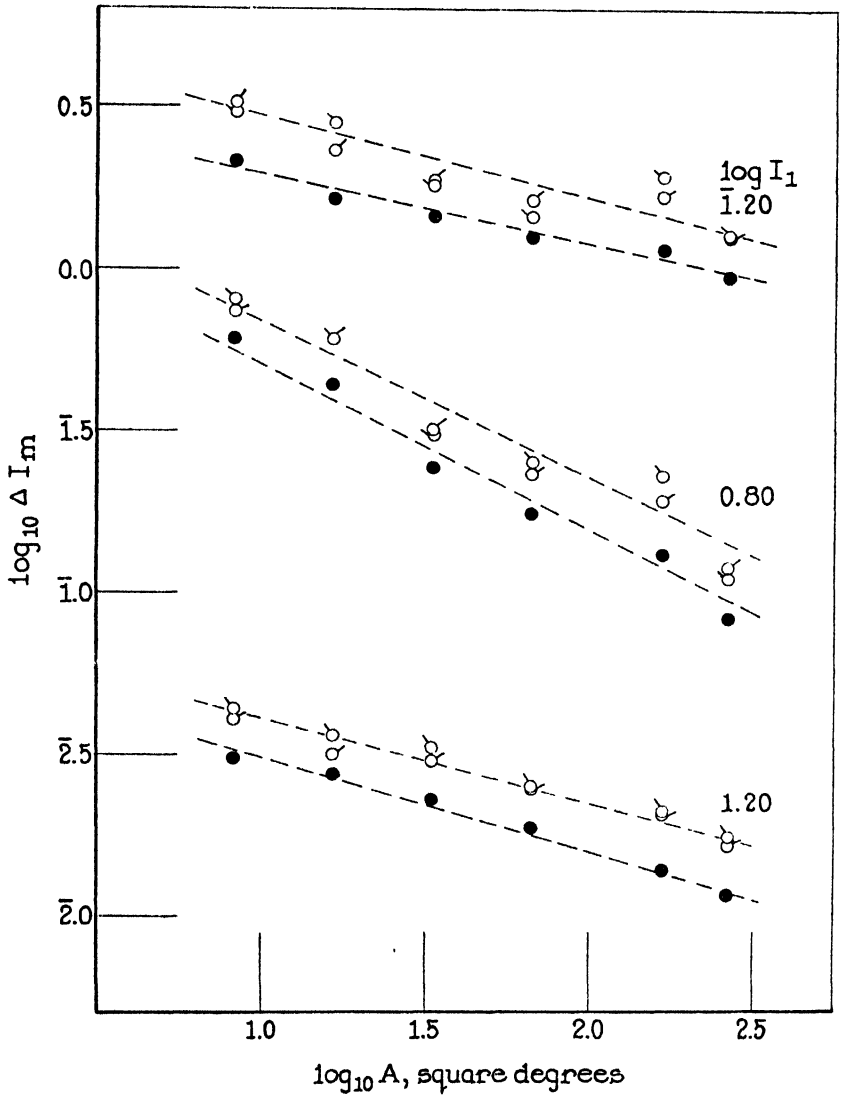

FIG. 11. Data of Table VII, homogeneous for the comparison of monocular with "binocular" differential thresholds; ΔI_m for right eye and left eye are indicated by directions of the tags on the open circlets; for binocular observations, by solid circlets. Central plots, at $\log I_1 = 0.80$, are for the method of Table II, the others for the method of Table I (see text).

0.255 when log area A is taken to base 10, for both the monocular and binocular measurements.

The mean difference between $\log\left(\frac{\Delta I_L + \Delta I_B}{2}\right)$ and $\log \Delta I_B = 0.46 \log_{10}$ unit; or 0.14 with A to base 2. The antilog of 0.14 = 1.38. For absolute thresholds (*cf.* also Lythgoe and Phillips, 1938) we have obtained 1.41 for the ratio of monocular to binocular excitatory intensities (Crozier and Holway, 1938-39 *b*), but we noted that for greater than zero values of I_1 the ratio fell below 1.4. The meaning of this ratio clearly is that duplex presentation enlarges the number of potentially excitable elements, and may double it; hence the fineness of statistical discrimination is increased, and the recognizable difference between I_1 and I_2 becomes smaller, together with its σ (*cf.* Crozier, 1936, *etc.*).

The ratio between $(\Delta I_L + \Delta I_B)/2$ and ΔI_B must obviously be in general an individual constant. Consider the case of any individual with defect in one eye, so that $\Delta I'$ is very much greater or less than $\Delta I''$; here the value of the ratio must be *less* than 1.4. It is also a fact (our unpublished data) that a large light image of *subliminal* intensity, placed on the retina of one eye can quite detectably influence the threshold stimulus as applied to the *other* eye.

None of the series in Table VII is homogeneous for the *form* of the area-ΔI function, since they involved successive use of each eye followed by the use of both at each area. We might expect, therefore, to find greater scatter of points in the graphs. However, the slopes of the lines as drawn (Fig. 11) agree well with those gotten respectively for the two types of experiments, in which (1) excited area is symmetrically enlarged on either side of the fovea (Z = 0.267, and 0.255), and (2) by increasing steps toward the fovea (Z = 0.591, and 0.495). For the data and conditions of Table VII, Z is in each case a little lower, which may well be accounted for by the difference in the procedures. This result is fully confirmed by another extensive series of measurements, involving larger areas, data for which are not given in this paper, which also gave identical values of Z for right and left eyes and for both together, but Z here = 0.24.

IX

Organic Variability

One of the first attempts to deal with the form of the function relating (over a limited range) differential sensitivity ($= 1/\Delta I$) and discriminatory precision ($= k/\sigma_{\Delta I}$) was made by G. E. Müller (1879); see also Troland

(1917). Others have been concerned with the relation from the standpoint of correlation methods (Thomson, 1912; Culler, 1927), showing that the two quantities are not independent but are directly related—at least over moderate ranges, and with non-homogeneous data. More recently the

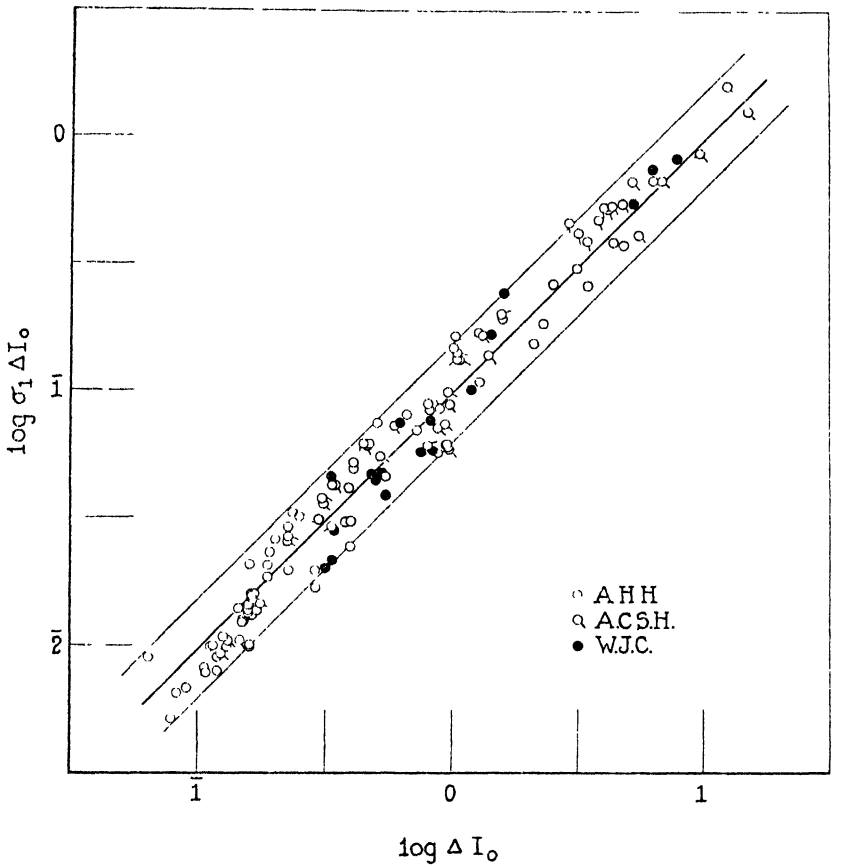

FIG. 12. The relation between ΔI_m and $\sigma_{1_{\Delta I}}$, for absolute thresholds (ΔI_0), for three observers (data in Crozier and Holway, 1938–39 *b*). Proportionality is direct (*i.e.*, the slope of the graph = 1). The line bisecting the area between the margins has 59 points above it, 60 below; in such non-homogeneous data log $\sigma_{\Delta I}$ is symmetrically ("normally") distributed at fixed ΔI; see text.

relation has been determined over wide ranges of intensities for several different sensory modalities (*cf.* Crozier, 1935; 1936; Upton and Crozier, 1936; Crozier and Holway, 1937; 1938; Holway and Crozier, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1936–39). In general, for all these cases, includ-

ing those in which a critical intensity has the meaning of a discriminated ΔI,

$$\sigma_{\Delta I} = k'(\Delta I_m + C),$$

and C is almost always $= 0$.

Each of the quantities $\sigma_{\Delta I}$ and ΔI_m is probably determined organically. Strictly speaking they are gotten under *identical* experimental conditions, since both depend on the same measurements. Their interrelationship is intrinsically a law of the organism under scrutiny.

The values of σ here dealt with are of the order of 10 per cent of ΔI_m. Lest these be mistakenly supposed to signify a comparatively large variation, we may point out that we deal with σ_1, the root-mean-square dispersion, not the S.D. of the *mean*. If computed in the more usual manner, giving the P.E. of the mean as a percentage of the mean adjusted intensity, which is of course a function of the number of observations averaged, $\sigma_1 = 0.1\ \Delta I_m$ here corresponds to *ca.* P.E.$_m < 0.3$ per cent of I_2.

We have already shown that $\sigma_{\Delta I} = k\ \Delta I$ for I_1 as variant (Crozier and Holway, 1937; 1938). If it can now be shown that the same law operates at any fixed level of I_1, but with A as variant, then it cannot be assumed that $\sigma_{\Delta I}(= k\,\sigma_{I_2})$ is determined by the magnitude of the stimulus (intensity). For a given I_1, the *amount of light* entering the eye *increases* with the size of the retinal image. If equation (5) holds, however, $\sigma_{\Delta I}$ should nonetheless *decrease,* as it is found to do.

Fig. 13 shows that this is the case. Each plotted point is an average of five measurements. The outer lines depict statistical limits: $\sigma_{\sigma\Delta I}$ is directly proportional to $\sigma_{\Delta I}$ itself. The solid line is drawn with a slope $= 1.0$; and, for A and λ as variants, $\sigma_{\Delta I}$ for any value of I_1 is seen to be directly proportional to ΔI. We shall deal in another place with the evidence showing that the same rule holds with exposure time as variant. This fact is consistent with the hypothesis that this relation describes an organic invariant (*cf.* Crozier and Holway, 1937).

In Fig. 12 we also give the variation data from our earlier experiments on absolute thresholds as a function of retinal position (Crozier and Holway, 1938–39 *b*). The data plotted in each assemblage do not form a homogeneous population of σ's; consequently we must expect (*cf.* Holway and Crozier, 1937 *a*) that bisection of the upper and lower limits of log σ, at any (every) level of ΔI_m will divide the band population into numerically equal halves, as is illustrated in the figure. Data which are *homogeneous* show that the σ-width of the band must be divided arithmetically, not the log σ width, to achieve this result (Upton and Crozier, 1936; Holway and Crozier, 1937*a*); this is a consequence of the fact that $\sigma_{\Delta I}$ *determines* ΔI_m. It is noteworthy that when $I_1 = 0$ (*i.e.*, for absolute thresholds) $\sigma_{1\Delta I_0}$ has the

same value for a given level of ΔI_0 as for $\sigma_{1\Delta I}$ with I_1 finite; consequently $\sigma_{1\Delta I}$ cannot be regarded as a constant fraction of I_1 or of I_2. The log σ intercept values at given levels of log ΔI_m for observer A.H.H. (Fig. 12), measuring the proportionality constant in the direct relationship between ΔI_m and $\sigma_{1\Delta I}$ (and thus inversely estimating the precision with which ΔI_m

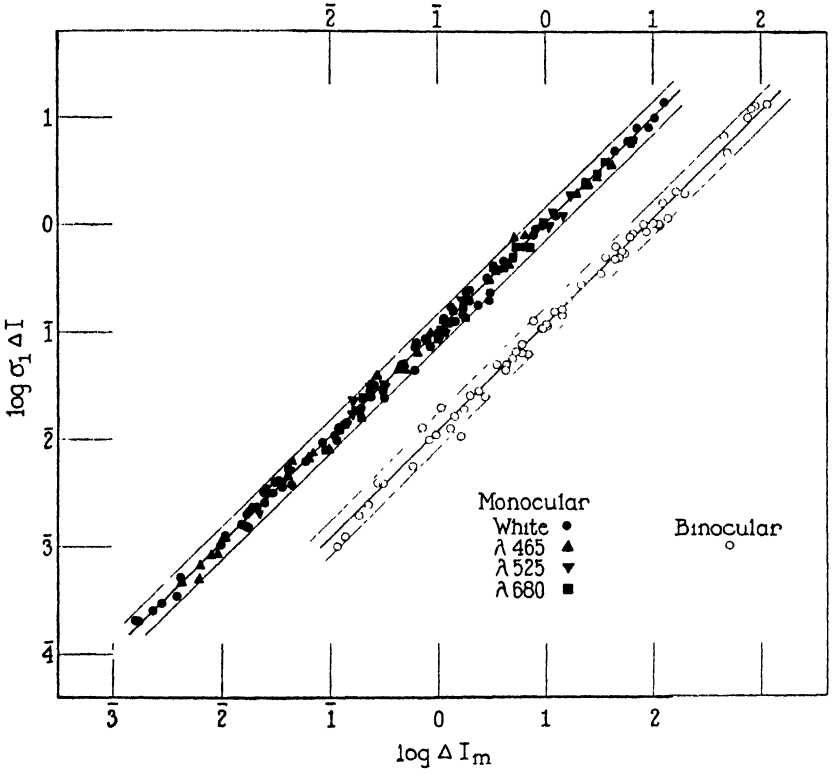

FIG. 13. The relation between ΔI_m and $\sigma_{1\Delta I}$ for ΔI at finite levels of I_1, with area and λ as parameters, is one of constant proportionality for an observer (data of Tables I, II, and V); for binocular observations (log ΔI scale above) the proportionality constant is a little higher.

is obtained), are $\bar{1}.02$ for the main monocular tests and $\bar{1}.07$ for the chief binocular tests discussed in the present paper. For an earlier series of experiments (*cf.* section III) the value $\bar{1}.26$ was obtained. It is of course conceivable that the 15 per cent increased precision in the newer experiments may represent the effect of experience ("practice"); but this is unlikely for several reasons: (1) there is no evidence of drift of precision during any one set of experiments extending over several weeks, and (2)

several series of tests by other methods, before and after this particular series, give higher values of the precision. These latter series have already been briefly considered elsewhere. For ΔI tests involving the method of successive comparison with a *linear* increase of $\tilde{I}_2$ (Holway and Hurvich, 1937), $\log \sigma_{1\Delta I}$ for $\log \Delta I_m = 0$ is $\bar{2}.99$ (*cf.* plot in Crozier and Holway, 1937); for a series involving photometric adjustments with increase of $\tilde{I}_2$ by inverse square of distance (Holway, 1937) $\log \sigma_{1\Delta I}$ is $\bar{1}.15$ (Crozier and Holway, 1937). In our series of absolute threshold (ΔI_0) measurements (white light; Crozier and Holway, 1938–39 *b*) it is $\bar{2}.98$ (Fig. 12); in a further series of relative threshold data it is $\bar{2}.93$; these two sets involved *logarithmic* increase of $\tilde{I}_2$. Thus there is no relationship between the precision and the mode of manipulation of the adjusted intensity, whether by logarithmic optical wedge, linear increase, or by use of the inverse square law, under the conditions of these experiments. It is accordingly difficult to conceive that the law of the scatter of ΔI_1 is determined by the character of the manipulative inaccuracies inherent in different kinds of operating procedure. In these cases cited a given small false adjustment of the manipulated distance of lamp or of travel of the wedge will obviously produce a change of intensity in the logarithmic case proportional to I, in the inverse square case proportional to $I^{3/2}$, and in the linear case an amount which is always constant. Therefore different laws would necessarily be obtained if the properties of $\sigma_{\Delta I}$ were governed by "observational error" of instrumental origin.

It is clear, we believe, that the findings made on the basis of predictions that the variation measured by $\sigma_{\Delta I}$ is organically determined by the tested individual are uniquely consistent with this position. Of these we may refer here to 2: the interrelationships between σ_F and σ_I for responses to visual flicker (Crozier, Wolf, and Zerrahn-Wolf, 1936–39), and the interdependence of $\sigma_{\Delta I}$ and of σ_{I_1} in ΔI measurements (Crozier and Holway, 1938). Further (unpublished) experiments in which, by means of *heterochromatic* matches, "ΔI" has been determined over a range of constant brilliances, show that the law for the behavior of $\sigma_{\Delta I}$ is predictably different depending upon which of the two differently colored lights is adjusted, although their modes of manipulation are identical.

X

SUMMARY

Measurements of ΔI as a function of retinal area illuminated have been obtained at various levels of standard intensity I_1, using "white" light and

light of three modal wave-lengths (λ465, 525, 680), for monocular stimulation and for simultaneous excitation of the two eyes ("binocular"), using several methods of varying (rectangular) area and retinal location, with control of exposure time.

For data homogeneous with respect to method of presentation,

$$\log \Delta I_m = -Z \log A + C,$$

where $\Delta I = I_2 - I_1$, A is area illuminated, and C is a terminal constant ($= \log \Delta I_m$ for $A = 1$ unit) depending on the units in which ΔI and A are expressed, and upon I_1.

The equation is readily deduced on dimensional grounds, without reference to specific theories of the nature of ΔI or of retinal area in terms of its excitable units. Z is independent of the units of I and A. Experimentally it is found to be the same for monocular and binocular excitations, as is to be expected. Also as is expected it is not independent of λ, and it is markedly influenced by the scheme according to which A is varied; it depends directly upon the rate at which potentially excitable elements are added when A is made to increase.

For simultaneous excitation of the two eyes (when of very nearly equivalent excitability), $\Delta \bar{I}_B$ is less than for stimulation of either eye alone, at all levels of I_1, A, and λ. The mean ratio $(\Delta \bar{I}_L + \Delta \bar{I}_R)/2$ to ΔI_B was 1.38. For white light, doubling A on one retina reduces ΔI_m in the ratio 1.21, or a little less than for binocular presentation under the same conditions. These facts are consistent with the view that the properties of ΔI are quantitatively determined by events central to the retina.

The measure $\sigma_{1\Delta I}$ of organic variation in discrimination of intensities and ΔI_m are found to be in simple proportion, independent of I_1, A, λ (and exposure time). Variability ($\sigma_{1\Delta I}$) is not a function of the mode of presentation, save that it may be slightly higher when both retinas are excited, and its magnitude (for a given level of ΔI_m) is independent of the law according to which the adjustable intensity I_2 is instrumentally controlled.

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TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT[1]

V. Xiphophorus, Platypoecilius, and Their Hybrids

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(Received for publication, March 24, 1939)

I

For various kinds of animals there are found[2] pronounced quantitative and qualitative differences in the dependence of visual excitability upon temperature. It has also been shown[3] that specific values of certain parameters descriptive of the form of the visual response contour (for reaction to flicker) behave as heritable invariants in cross-breeding experiments involving teleosts of the genera *Xiphophorus* and *Platypoecilius*. The question arises, do these constants differ in their dependence upon temperature?

Parameters which are heritable must be considered to express determinate properties of distinct assemblages of elements. The fact that the function efficiently describing the relation of critical intensity to flash frequency is a probability integral[4] in log I raises a number of significant queries. The use of this particular function is required for two reasons: it describes the course of the data, thus far uniquely well; and its parameters ($F_{max.}$; $\tau' = \log I$ at inflection; and $\sigma'_{\log I}$) have been shown to experimentally exhibit appropriate properties. $F_{max.}$ and $\sigma'_{\log I}$ are the measures which show genetic invariance numerically. They are expressions of essentially statistical attributes of the assemblages of elements concerned. When temperature is varied they are found to remain constant. Hence it cannot be assumed that reaction velocities are directly concerned in their deter-

[1] Previous reports in *J. Gen. Physiol.*, 1936-37, **20**, 393, 411; 1938-39*a*, **22**, 311; 1938-39*b*, **22**, 487; 1938-39*c*, **22**, 795. *Proc. Nat. Acad. Sc.*, 1938, **24**, 216; 1939*a*, **25**, 78; 1939*b*, **25**, 171; 1939*c*, **25**, 176.

[2] *Proc. Nat. Acad. Sc.*, 1938, **24**, 216; 1939, **25**, 78. *J. Gen. Physiol.*, 1938-39*a*, **22**, 311; 1938-39*b*, **22**, 487; 1938-39*c*, **22**, 795.

[3] *J. Gen. Physiol.*, 1937-38, **21**, 17; 1938-39, **22**, 463. *Proc. Nat. Acad. Sc.*, 1937, **23**, 516; 1938, **24**, 542.

[4] *Proc. Nat. Acad. Sc.*, 1937, **23**, 71. *J. Gen. Physiol.*, 1938-39, **22**, 311, 451.

mination. The abscissa of inflection, however, is dependent on temperature, and in such a way, quantitatively, as to require the assumption that the excitability ($1/I$; F fixed) is governed by the speeds of intrinsic reactions common to all the elements concerned in the production of a given reaction contour.

This situation permits a rather definitive test of two distinctly different questions. The first has to do with the proposition[5] that the characteristically duplex curves of visual performance obtained with vertebrates are of a form which permits the deduction of kinetically different modes of excitability for retinal rods and cones. The second concerns the more general conception of the nature of genetic differences. The potent evidence which can be obtained by way of genetic tests of organic invariance[6] indicates that a connection may be theoretically important and empirically significant. We have examined from this standpoint the dependence of response to flicker upon temperature in *Xiphophorus*, *Platypoecilius*, and their F_1 hybrids.

II

The technic of the tests has been described in other cases.[1] To favor homogeneity of observational conditions the number of animals tested has been reduced to the point where it has been possible to obtain very careful readings of critical intensities upon sets of *Xiphophorus* (*X.*), *Platypoecilius* (*P.*), and F_1 on the same day, under the same general conditions, within a space of several hours. Three observations were taken on each individual at each flash frequency (F) used, at each temperature. Observations on any one day were taken at the same temperature. The order of the temperatures used was essentially random, and included adequate check determinations at certain temperatures. Of *X. montezuma*, 5 individuals were used, of *P. maculatus*, 5; and of F_1, 2. The essential equivalence of individuals in any one of these stocks[1] obviates the necessity of large numbers of individuals, as indeed the present results also prove. This could only be justified, however, on the basis of adequate experience with these animals. The variation of the readings supplies an efficient check. The individuals used had been represented in our earlier experiment with these forms.[7]

Determinations of critical intensities were made after several hours' dark adaptation in a thermostat[8] at the desired temperature. During the comparatively brief interval

[5] *Cf.* Hecht, S., *Physiol. Rev.*, 1937, **17**, 239, and much earlier work there summarized. *Harvey Lectures*, 1937–38, **33**, 35.

[6] *J. Gen. Physiol.*, 1929–30, **13**, 57, 81; 1931–32, **15**, 421; 1936–37, **20**, 111; 1937–38, **21**, 17; 1938–39, **22**, 463.

[7] *Proc. Nat. Acad. Sc.*, 1938, **24**, 542. *J. Gen. Physiol.*, 1938–39, **22**, 463.

[8] *Cf. J. Gen. Physiol.*, 1932–33, **16**, 757. We are under obligation to the Trustees of the Elizabeth Thompson Science Fund for a grant to one of us which was used for the construction of one of our thermostatic tanks.

out of the thermostat required for an observation the aquarium containing a fish (in 250 ml. water), the temperature of the container, and probably that of the fish, changed a little. The temperature listed in the tables (I and II) has been corrected for this change, so as to give the mean temperature during the period of observation. This never differed by >0.3° from the adaptation temperature. While we naturally regret this source of inaccuracy, it has been impossible to thermostat the optical system employed for the observations, or the whole dark room. At the same time it is to be noticed that the temperature change in the fish was probably slower than in the aquarium water; and that the quantitative result obtained (Fig. 2) would be by this factor if anything slightly but definitely improved if the correction could really be applied in a more adequate way.

The behavior of the different lots of individuals used is not quite the same. *X. montezuma*, *P. maculatus*, and F_1 (or H'') have been described previously.[7] We have to add certain points concerning relations to temperature, and also some comments on the F_2 individuals obtained from our F_1's (*cf.* Section VI).

X. montezuma is less quiet than *P. maculatus*. Quite young individuals are on the whole not so restless, but are difficult to observe at low illuminations, and consequently older individuals were used. Their reactions are clearer at low flash frequencies. With higher temperature the critical reactions become sharper and more vigorous. At lower temperatures the end-point must be approached with particular care to avoid overshooting of the critical intensity. At $F = 25$ restlessness increases with rise of temperature.

P. maculatus is steadier, and at low illuminations the observation of the marginal response to flicker presents no difficulties at any temperature. At $F = 25$, and particularly at higher temperatures, the platys tend to push against the cylinder wall, nose down, and may exhibit a general activity—due in large part to the light—upon which the forced reaction to discriminated flashes is superimposed during quiescent intervals.

The F_1 hybrids are more like *X.* than like *P.* in their behavior. Even after 6 months under observation they are comparatively "wild;" readings are taken during periods of quiescence. At higher temperatures restlessness is greater, at $F = 25$ especially.

F_2 hybrids were obtained (several broods) from 1 F_1 ♂ and 2 F_1 ♀♀. The age differences in the lot used were 5 to 6 months (lengths 14 to 30 mm.). They are much more quiet than F_1. All tests with these were made at 21.5°C. Up to $F = 10$ responses were quite clean and sharp, mostly as a motor jerk in the direction of rotation of the stripes. Above $F = 10$ these animals stay at the periphery of the jar and respond by moving slowly (but with rapid fin motions) in the direction of passage of the stripes; the responses are more difficult to recognize. In general the behavior is much more like platy than like *montezuma*, in contrast to F_1, but this may easily be due in part to the fact that they were subjected to the laboratory routine from an earlier age.

It is to be noted that small and large individuals of any one of these types are quantitatively indistinguishable in their values of critical intensities. This was most elaborately shown with F_2 (Section VI), but was found also for *X. montezuma*.

The summarized observations are given in Tables I and II. Our plan was to secure data at a low flash frequency within the purely "rod" section of the duplex flicker response contour, and also at a much higher frequency within the purely "cone" segment of the curve (*cf.* Fig. 1). For this purpose

TABLE I

Mean critical intensities and the dispersions of the individual values (as log millilamberts) for response to visual flicker at the low flash frequencies (per second) indicated, for *X. montezuma*, *P maculatus*, and their F_1 hybrids, at various temperatures; flash cycle with light and dark intervals equal. For *X.* and *P.*, $N = 5$ individuals each; for F_1, $N = 2$; 3 readings on each individual at each temperature. (Values in parentheses are from parallel tests with other lots of individuals.)

	$F = 4.0$		$F = 2.5$		$F = 3.0$	
$t°_{corr.}$	*X. montezuma*		*P. maculatus*		F_1, *X.* × *P.*	
	log I_m	log P.E.$_{.1I}$	log I_m	log P.E.$_{.1I}$	log I_m	log P.E.$_{.1I}$
12.7	$\bar{6}.8913$	$\bar{7}.1577$	$\bar{5}.4098$	$\bar{7}.8028$	$\bar{5}.0846$	$\bar{7}.6850$
15.4	$\bar{6}.7793$	$\bar{7}.0569$	$\bar{5}.3023$	$\bar{7}.4554$	$\bar{5}.0330$	$\bar{8}.9227$
17.2	$\bar{6}.7065$	$\bar{8}.8945$	$\bar{5}.2222$	$\bar{7}.3699$	$\bar{6}.8935$	$\bar{7}.0733$
19.2	$\bar{6}.6522$	$\bar{8}.9018$	$\bar{5}.2028$	$\bar{7}.6761$	$\bar{6}.8710$	$\bar{8}.7894$
21.5	$\bar{6}.5942$	$\bar{8}.8892$	$\bar{5}.1274$	$\bar{7}.5987$	$\bar{6}.8197$	$\bar{8}.3665$
	$\bar{6}.5936$	$\bar{8}.9199$	$\bar{5}.1202$	$\bar{7}.6028$	$\bar{6}.7543$	$\bar{8}.8189$
	($\bar{6}.5891$	$\bar{8}.7059$)			$\bar{6}.7487$	$\bar{8}.3705$
	($\bar{6}.6052$	$\bar{7}.2299$)			$\bar{6}.9534$	$\bar{8}.8235$
23.9	$\bar{6}.4823$	$\bar{8}.9287$	$\bar{6}.9827$	$\bar{7}.1813$	$\bar{6}.6981$	$\bar{7}.1383$
			$\bar{5}.0255$	$\bar{7}.5883$		
27.2	$\bar{6}.4108$	$\bar{8}.7950$	$\bar{6}.8764$	$\bar{8}.9577$	$\bar{6}.5441$	$\bar{8}.7633$
29.7	$\bar{6}.3126$	$\bar{8}.6640$	$\bar{6}.8073$	$\bar{7}.1562$	$\bar{6}.5222$	$\bar{7}.0043$
32.7	$\bar{6}.2462$	$\bar{8}.6842$	$\bar{6}.7786$	$\bar{8}.6406$	$\bar{6}.5046$	$\bar{8}.6850$
35.6	$\bar{6}.1697$	$\bar{8}.3257$	$\bar{6}.6111$	$\bar{8}.8539$	$\bar{6}.3222$	$\bar{8}.6630$

TABLE II

As in Table I, but at flash frequency $F = 25$ per second.

	$F = 25$					
$t°_{corr.}$	*X. montezuma*		*P. maculatus*		F_1, *X.* × *P.*	
	log I_m	log P.E.$_{.1I}$	log I_m	log P.E.$_{.1I}$	log I_m	log P.E.$_{.1I}$
12.7	$\bar{1}.7646$	$\bar{2}.0966$	0.5831	$\bar{1}.0726$	0.3003	$\bar{2}.9697$
15.4	$\bar{1}.6717$	$\bar{2}.0730$	0.4735	$\bar{2}.9373$	0.1661	$\bar{2}.3893$
17.2	$\bar{1}.5786$	$\bar{3}.6603$	0.3804	$\bar{2}.8353$	0.0856	$\bar{2}.2679$
19.2	$\bar{1}.5345$	$\bar{3}.8193$	0.3495	$\bar{2}.7761$	0.0430	$\bar{2}.2536$
21.5	$\bar{1}.4571$	$\bar{3}.5317$	0.2896	$\bar{2}.6109$	$\bar{1}.9999$	$\bar{2}.0367$
	$\bar{1}.4639$	$\bar{2}.1501$	(0.3002)		$\bar{1}.9880$	$\bar{2}.5750$
	($\bar{1}.4700$	$\bar{3}.8653$)	(0.2833)			
	($\bar{1}.4598$	$\bar{3}.9677$)	(0.3107)		0.0020	$\bar{3}.9943$
	($\bar{1}.4708$	$\bar{3}.9719$)	(0.2911	$\bar{2}.8185$)		
23.9	$\bar{1}.3769$	$\bar{3}.6897$	0.2030	$\bar{2}.2894$	$\bar{1}.9046$	$\bar{2}.2191$
27.2	$\bar{1}.2617$	$\bar{3}.7205$	0.0962	$\bar{3}.9925$	$\bar{1}.8211$	$\bar{3}.7356$
29.7	$\bar{1}.1940$	$\bar{3}.5861$	0.0233	$\bar{2}.2403$	$\bar{1}.7361$	$\bar{3}.6516$
32.7	$\bar{1}.1326$	$\bar{3}.4407$	$\bar{1}.9357$	$\bar{2}.1278$	$\bar{1}.6575$	$\bar{3}.6399$
35.6	$\bar{1}.0342$	$\bar{3}.3699$	$\bar{1}.9916$	$\bar{3}.8785$	$\bar{1}.5541$	$\bar{2}.3653$

we chose for *X.*, *P.*, and F_1 respectively the levels $F = 4, 2.5$, and 3 (*cf.* footnote 7), and for all of them $F = 25$. The fact that for each of the three forms the difference $\Delta \log I_m$ between mean critical intensities at the high and low flash frequencies is independent of temperature shows directly that the *form* of the $F - \log I_m$ contour is independent of temperature.[1] With the sunfish *Enneacanthus*[8a] we discovered that for the low-F segment of the curve there occurs a dislocation with respect to the upper (cone) part, at *ca.* 20°C. With the fishes herein concerned this is not found.

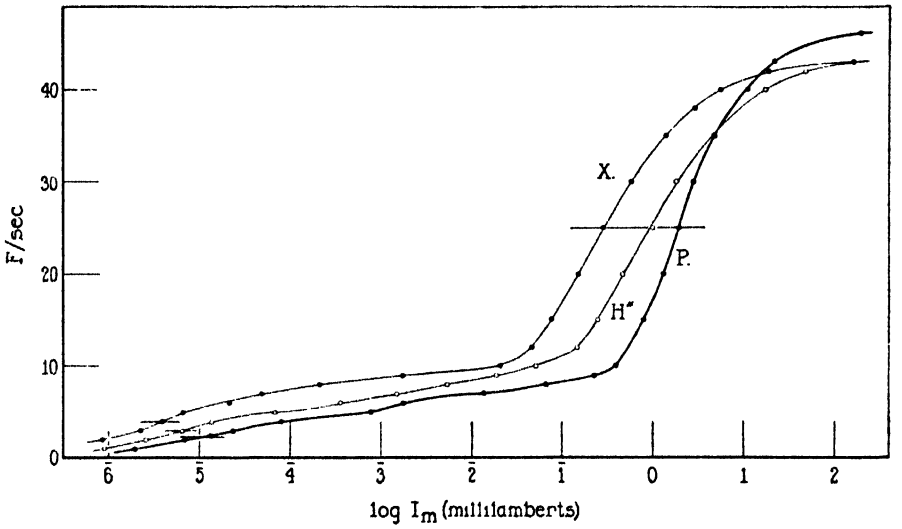

FIG. 1. Flicker response contours for *X. montezuma* (*X.*), *P. maculatus* (*P.*), and their F_1 hybrids (*H"*). Temperature, 21.5°; flash cycle with light time = dark time; *cf.* Crozier, W. J., and Wolf, E., 1938–39, *J. Gen. Physiol.*, **22**, 463. The horizontal lines indicate the levels of F at which observations of dependence on temperature were made (Tables I and II).

III

The data of Tables I and II are plotted in terms of the Arrhenius equation $\ln k = \exp. (-\mu/RT) + c$, in Fig. 2. For this purpose it has been considered that the proper measure of excitability is to be taken as $1/I_m$. The fact that F *vs.* $\log I_m$ is the fundamental descriptive function[4] is no obstacle. F *vs.* $\log I_m$ is a summation curve of neural effects produced; at a given level of F, with other relevant conditions fixed, these effects are elicited by flash intensity I. The capacity of intensity I to arouse effects

[8a] *Proc. Nat. Acad. Sc.*, 1939, **25**, 78. *J. Gen. Physiol.*, 1938–39, **22**, 487.

adequate to occasion response is presumably governed by the velocities of metabolic events in the neural units concerned. If a particular kind of

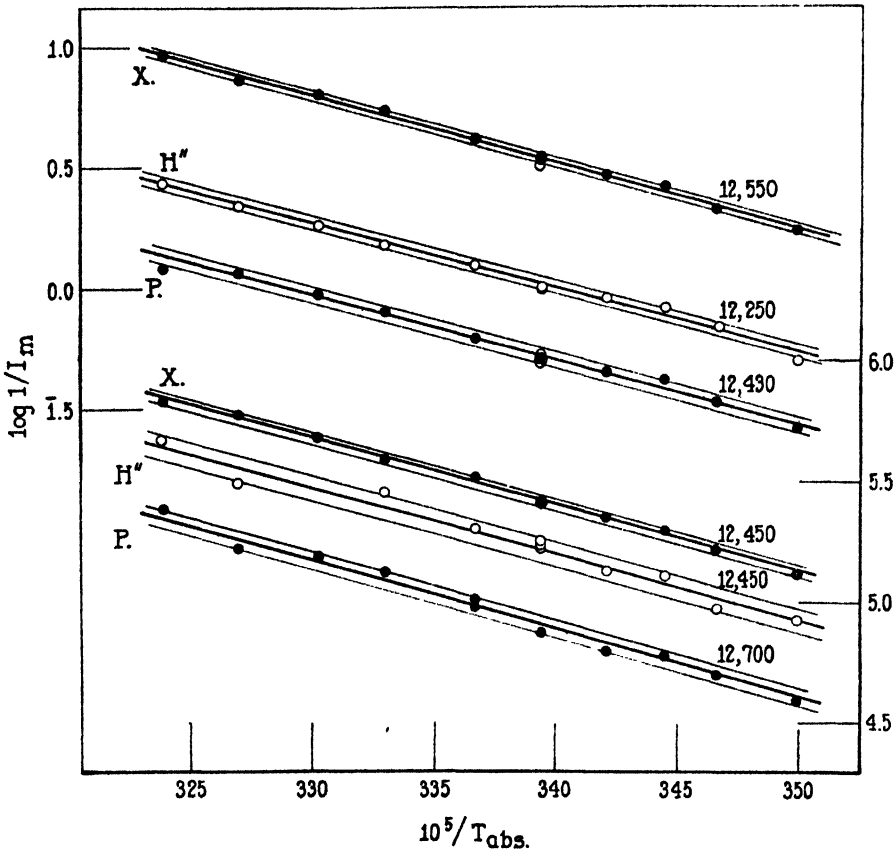

FIG. 2. Data in Tables I and II, plotted to show rectilinearity in terms of

$$\ln (1/I_m) = exp. (-\mu/RT) + C,$$

for each flash frequency. The slopes of the lines, and consequently μ, are not distinguishably different; consequently $\mu = 12{,}400$ is independent of F.

The three upper lines refer to the cone parts of the $F - \log I$ curves, the three lower to the rod parts (*cf.* Fig. 1).

The constant relative scatter of $1/I_m$, of course, depends in part on the number of observations in each series, but there are indications that it is intrinsically *lower* for X. than for P.

velocity plays a controiling rôle, and if it is of a physicochemical type sufficiently simple, it may be expected to reveal itself through the dependence of critical flash intensity on temperature. This intensity will vary

inversely with the velocities of the excitability process if its reciprocal is a rational measure of "excitability." The simplicity or complexity of the governing processes is something to be determined from the properties of the data, not by anterior convictions about the nature of events in organisms. The graphs in Fig. 2 show that $1/I$ is described by the Arrhenius equation and that for *X.*, *P.*, and F_1 μ for $1/I_m$ is demonstrably equivalent, both in the rod and in the cone segments of the F-log I contour.

The values of the temperature characteristic μ in Fig. 2 are, from above downward, 12,550; 12,400; 12,430; 12,450; 12,600; 12,700 (mean = 12,510 $\pm$ 30). These values do not differ significantly. They are obtained from the slopes of the lines adjusted to give the best reasonable descriptions of the dispositions of the points, with particular reference to the fact that in each series of readings σ_I is[1] directly proportional to I_m, independent of temperature. If, because of lag in drift of internal temperature during observations at temperatures much above or much below that of the room, the values of $t^\circ_{corr.}$ in Tables I and II have been over-corrected, the rectilinearity of the graphs in Fig. 1 would be somewhat improved and the value of μ would be slightly less (not below 12,300).

IV

For different animals[1] thus far investigated the values of μ obtained for $1/I_m$ are quite diverse. This is in correspondence with the fact that one must presume sunfish, turtle, dragon-fly nymph, and similarly unlike forms to be the embodiments of quite dissimilar physicochemical systems. Our genera *X.* and *P.* (both placed by taxonomists in the family *Poeciliidae*) are less unlike in this respect, since they can interbreed and produce fertile offspring. In the other forms mentioned not only are the values of μ different, and also different from the 12,500 for *X.* and *P.*, but also the types of physicochemical organization of visual excitability revealed by the occurrence of critical temperatures.[1] These facts provide a forceful empirical argument for the analytical significance of μ as a quantitative index of temperature dependence. One aspect of this matter deserves renewed comment. The argument we have used implies that for response at a low F the velocity of the process for which I is responsible is required to be much less than at a high F. In the present observations μ is independent of the magnitude of this presumptive velocity, over a range of I_m corresponding to a factor of 250,000 (*cf.* Tables I and II; Fig. 1)—much greater even than in our earlier observations on *Enneacanthus*.[8a]

This sort of homogeneity can result if the constant μ has the kind of meaning envisaged in the hypothesis that it represents the activation

energy of a governing catalyst,[9] and if the organic elements concerned in the eventuation of the index response constitute a truly homogeneous population (aside from statistical dispersion). An objective verification of this requirement is given by the dispersions of the measurements of critical intensities. No persistent differences in the excitabilities of the individuals in any one set were detectable; this is the condition already found in other work with fishes.[1] Fig. 3 shows that the relation between $P.E._{1_{I_1}}$ and I_m is rectilinear, when change of I_m is induced at fixed levels of F by altering

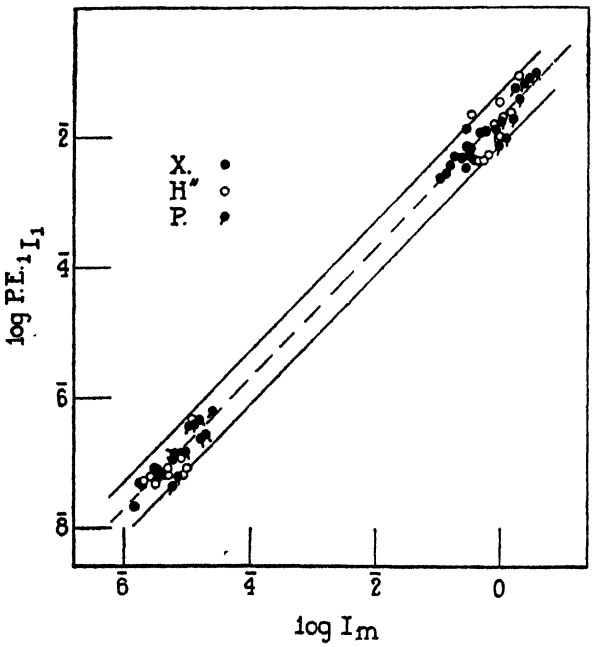

FIG. 3. The variation of I_1 is directly proportional to I_m for *X.*, *P.*, and *H″* when temperature is varied at fixed levels of F (the log-log slope = 1).

the temperature. The proportionality constants are identical with those found[10] in previous observations with these forms at constant temperature. When it is independently demonstrable that in experiments of this kind more than one process is involved in the control of the critical intensity[11] the variation of I_m when temperature is the variant does not exhibit these properties. Since the three sets of individuals providing the data in Fig. 3

[9] *J. Gen. Physiol.*, 1938–39, **22**, 311; *cf.* 1924–25, **7**, 189, etc.
[10] *J. Gen. Physiol.*, 1938–39, **22**, 463.
[11] *J. Gen. Physiol.*, 1938–39, **22**, 795.

are not homogeneous, we, of course, find that the division of the band into halves with equal numbers on either side of a central line requires the bisection of the log P.E. span, not of arithmetic P.E. This corresponds to the effect of independent fluctuations of a visual performance function as a whole in series of determinations with one individual over a period of time.[12]

For the sunfish *Enneacanthus* we found for $1/I_m$ μ = 8,200 (12 to 20°) and μ = 14,400 (20 to 30°); for *Pseudemys* (turtle), μ = 27,000 (11 to 29.5°) and μ = 12,400 (29.5 to 36°); incomplete experiments with *Fundulus* gave (above 20°) μ = *ca.* 12,600; with *Anax* there can be distinguished a controlling process with μ = 19,200 (8.5 to 35°). The forms of the $F - \log I$ contours for these animals are quite different. The types of dependence on temperature also differ, as shown by the occurrences of critical temperatures. In this respect the control of visual excitability resembles that for such properties as the frequency of the heart-beat in various animals, and is not inconsistent with the view that the excitability measured is governed by the speeds of common cellular metabolic processes. With the exception of μ = 8,200 at lower temperatures with *Enneacanthus*, the temperature characteristics thus far found are not those typically associated with respiratory oxidations. The indication is that with such forms as *X.* and *P.* over a considerable range the level of respiratory oxidations in the neural elements concerned might affect the value of I_m with $t°$ constant, but without modifying its temperature characteristic.

That in all these cases rise of temperature lowers I_m, and that the values of μ are very much higher than permits assigning them to photochemical reactions, is quite consistent with the conception that the determination of marginal response to flicker is dependent on central nervous discrimination[1] between the results of flashes and their after effects. We are not called upon to give a specific interpretation for the value of μ obtained in the present experiments, but it may be pointed out that μ = 12,200+ has been typically found for frequencies of various (non-respiratory) movements of arthropods,[13] and also in connection with heart-beat frequencies for which μ = 8,300, 14,400, 20,000, and 27,000, have also been found.[14] No interpretation of the specific causation of μ = 12,400 is required for our present purposes, but merely the fact that it is a valid constant (Fig. 2) and that its value is so high.

[12] *Proc. Nat. Acad. Sc.*, 1937, **23**, 23; 1938, **24**, 130.

[13] *J. Gen. Physiol.*, 1926–27, **7**, 123, 565; 1928–29, **9**, 49; 1929–30, **10**, 227.

[14] *E.g.*, *J. Gen. Physiol.*, 1927–28, **11**, 349.

V

For the interpretation of the shapes of flicker response contours it has been held desirable to push to the plausible limit the assumption that they are governed by the kinetics of processes limiting or determining primary peripheral excitation. The logical necessity for this is not especially notable, since it should be obvious that the responses providing the basis for the measurements are not actually given by the retina but are determined by the performance of the animals as reacting systems. Descriptions of the forms of the rod and "cone" branches of the flicker response contours for vertebrates have been given in terms of photochemical reaction systems presumed or deduced to characterize differences between the excitatory capacities of retinal rods and cones. These differences provide, for any one kind of animal, an "internal" situation posing a test of these general ideas. The essential point is that for such forms as sunfish (*Enneacanthus*), *Xiphophorus*, and *Platypoecilius* it is necessary to assume on this theory[5] that the photochemical pseudostationary state in the rod elements is specifically different from that in the cones, as we have already pointed out.[15] As deduced from the supposed applicability of Talbot's law to the situation at marginal flicker, with the assumption that the velocities of photochemical and dark processes are critically unbalanced at the point of marginal response, the equation for a flicker contour takes the form[16, 5]

$$pKI = F^n/(F_{max.} - F)^m$$

where p = the percentage light time in the flash cycle, K is a composite constant, and n and m are the apparent orders respectively of the dark and light processes. The equation fails to predict even the correct direction of the shift of the curve with change of temperature,[17] let alone the form of the temperature function. It makes erroneous predictions as to the dependence of the position and other properties of the flicker contour when the light time fraction is altered.[18] Nevertheless it is desirable to show that in a purely qualitative sense this equation fails to interpret the flicker response contour in any single type of vertebrate.

[15] *J. Gen. Physiol.*, 1935–36, **20**, 363, 411; 1937–38, **21**, 17.

[16] Hecht, S., Shlaer, S., and Smith, E. L., Intermittent light stimulation and the duplicity theory of vision, in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1935, **3**, 237.

[17] *J. Gen. Physiol.*, 1936–37, **20**, 393, 411; 1938–39, **22**, 311, 795. *Proc. Nat. Acad. Sc.*, 1938, **24**, 216; 1939, **25**, 78.

[18] *J. Gen. Physiol.*, 1937–38, **21**, 313, 463. Ross, R. T., *J. Gen. Psychol.*, 1938, **18**, 111.

The question cannot be decided by curve fitting to observations under fixed conditions. One reason is that for duplex performance curves (*a*) the rod curve is usually much too small, and in some cases (*e.g.* man, newt[19]) too complexly involved with the cone segment, and (*b*) the larger cone part cannot be directly tested in the lower 20 per cent of its range. For such duplex contours it has been found necessary to conclude that the mechanism of excitability includes systems of processes in which the *orders* (*i.e.*, n and m in the equation) of the reactions differ for rods and cones, in the same animal. If the dependence of critical intensity upon temperature proves to be quantitatively the same, however, for rod and cone curves it cannot be assumed, nor on any ground asserted, that the physicochemical basis for visual performance is different in the rod and cone elements. This is not the same thing by any means as stating that they are identical. If the physicochemical basis for primary excitation of retinal rods and cones is different in essential particulars, this difference is not involved in the differing shapes of the two branches of the flicker response contour. And the chemical kinetics of the primary excitation reactions therefore cannot be deduced from the form of the curve.

For *Enneacanthus*, *X.*, *P.*, *H'*, and *H''*, the application of the photostationary state equation provides a passable description of the rising phase of the $F - \log I$ contour.[20] For *Triturus* (newt)[19] and certain others it does not. Nor in any case does it give anything like an acceptable interpretation of the intermediate region of the F-log I curve where the putative rod and cone branches overlap. The upper cone segments are in general rather well described. For those cone segments which are of low slope and much involved in the rod part,[21] the description applies only to the highest levels of F. For much more completely exposed cone curves the use of the equation gives actually no better a description than is given by a logistic in log I (*i.e.*, $n = m$). For completely isolated simplex visual performance curves, rod or cone, the equation does not fit at all.[22] In these cases values of n and m (apparent reaction orders for opposed dark and light reactions) are chosen as *1* or *2*. In different animals the values which have to be selected differ for the rod and cone branches. In any one animal they also differ in this way.

[19] *J. Gen. Physiol.*, 1937–38, **21**, 203. *Proc. Nat. Acad. Sc.*, 1938, **24**, 125.

[20] *J. Gen. Physiol.*, 1935–36, **20**, 363; 1937–38, **21**, 17. Hecht, S., 1938, *J. Appl. Physics*, **9**, 156. *Harvey Lectures*, 1937–38, **33**, 35.

[21] Data on *Fundulus*, in course of publication.

[22] *Proc. Nat. Acad. Sc.*, 1938, **24**, 125, 216, 538. *J. Gen. Physiol.*, 1938–39, **22**, 311, 795.

Since the flicker response contours for *Enneacanthus*, *X.*, turtle, *Anax* and the like exhibit differences in their shape constants, and since μ for $1/I$ is likewise different, it could be entertained as a possibility (but not proved) that with these forms, differences in the chemical (including photochemical) kinetics of the excitatory processes are involved in producing the differences in shapes of the contours. The argument is completely independent of any specific assumptions whatever concerning the form which the mass action kinetic equations employed to describe the data may be forced to take. But the same kinds of differences in shape constants occur in the rod and cone segments for a single kind of animal, as in *X.*, *P.*, *H'*, *H''*, and *E.*[8a] Yet for each of these cases (excepting *H'*, not tested) the temperature characteristics (μ) for $1/I_m$ at fixed F are identical.

Consequently one is not permitted to assume that in any of these fishes the chemistry of the excitability situation is different in the rod and cone branches of the intensity function. The alternative is to conclude that the excitabilities concerned in determining the quantitative properties of the data are not the excitabilities of the primary rods and cones in the retina. However different the intrinsic basis of photic excitation may be in the several kinds of retinal elements, these differences cannot be characterized from the properties of the two branches of the flicker contour, and the photochemical kinetics of the primary excitation cannot be deduced from its shape.

This conclusion is amply reinforced by the fact that the shape constants for cone and rod parts in *X.* and *P.* differ, in the same manner as for other forms, yet for their quite dissimilar contours μ is identical. It cannot be assumed that the chemistry of excitability is different, for either the rod population or the cone, in *X.* and *P.* Moreover, the shape constants are heritable, as the data on F_1 prove, yet μ for rod and cone parts of the contour is again the same. Therefore, μ is independent of the shape constants.

The general conception that the quantitative properties of data of visual performance are not determined at the periphery,[23] and consequently do not reflect quantitative properties of the primary receptors, is obviously supported by a number of additional facts not otherwise accounted for save by the use of various extra and very dubious assumptions. Thus the result of induced variations in the availability of vitamin A is to synchronously modify both rod and cone thresholds in the same direction;[24] the same is

[23] Crozier, W. J., and Holway, A. H., 1938–39, *J. Gen. Physiol.*, **22**, 341.

[24] Haig, C., Hecht, S., and Patek, A. J., Jr., *Science*, 1938, **87**, 534. Hecht, S., and Mandelbaum, J., *Science*, 1938, **88**, 219. Wald, G., Jeghers, H., and Arminio, J., *Am. J. Physiol.*, 1938, **123**, 732.

true of changes in pressure of atmospheric oxygen.[25] At the same time, retinal adaptation may occur without detectable change in concentration of visual purple,[26] supposedly the basis for the distinction between rod chemistry and that of cones[27] and so far as now known the only basis for the peripheral involvement of vitamin A in photic excitability.[28]

The point has frequently been made that visual performance clearly involves peripheral as well as central factors. This is obvious enough, but the problem at the moment is to decide (*a*) whether the specific properties of the data, the essential invariant indices of performance capacity, are determined peripherally or not; and (*b*) which (if any) of the measures of performability have any connection with kinetic chemical features of excitability. It is to be noted that the form of the F-log I contour is certainly not specific,[29] as shown by the fact that a probability integral in the log of the independent variable applies to a great variety of dynamically similar but otherwise unlike situations; moreover experimental tests of the three parameters show that they have concordant properties in these cases. This formulation applies where one deals with the contributions summated from many varying elements, and if suitable separate determinations could be made of purely retinal and purely "central" phenomena they would pretty certainly be found to follow the same form of law as a function of intensity. Likewise, as other work has shown,[30] and as general considerations have indicated to be likely,[31] the same formulation is definitely applicable to various kinds of measures of visual performance. For these reasons it is already improbable that the quantitative properties of the data will tell anything about the kinetics of specific types of peripheral excitation. Decision as to the central or peripheral origin of the quantitative properties of the data must depend upon the use of other criteria.[32]

It might still be suggested that some sort of "limiting case" could be found in which, despite the passage of impulses from receptors to muscles involved in obtaining the data, we might have to do with a simple one-to-one correspondence between excited end-organ units and central units. It is to be pointed out, however, that, in such a case, we would still have to deal

[25] *Cf.* McFarland, R. A., and Evans, J. N., 1939, *Am. J. Physiol.*, **127**, 37.

[26] Granit, R., *J. Physiol.*, 1938, **94**, 430.

[27] Hecht, S., *Physiol. Rev.*, 1937, **17**, 239.

[28] Wald, G., *J. Gen. Physiol.*, 1935–36, **19**, 351; 1937–38, **21**, 795.

[29] *Proc. Nat. Acad. Sc.*, 1937, **23**, 71.

[30] The interpretation of the time and intensity function, and of photic adaptation, to be discussed elsewhere.

[31] *J. Gen. Physiol.*, 1935–36, **19**, 503.

[32] *Proc. Nat. Acad. Sc.*, 1936, **22**, 412; 1937, **23**, 23, 71.

with the fact that the units of the end result are not the same as those of the initial excitation. We are stressing the inescapable circumstance that the units involved are necessarily in terms of $\Delta E_1/\Delta I$, where E_1 is primary effect produced and I is intensity (or other independent evoking variable). If the units of end result R are $\Delta R/\Delta I$, then we have to recognize that if E_1 is in terms of impulses per unit time or something of that sort then when we put

$$\Delta R/\Delta I = K\,\Delta E_1/\Delta I$$

and eliminate ΔI we still must reckon with the complex dimensional constant K, and proof is required that it is independent of I and E_1 before we can argue for the direct interpretation of R in terms of the initiating mechanism of excitation.

VI

The shape constants ($\sigma'_{\log I}$), maxima, and abscissae of inflection (τ') for the F-log I_m curves differ markedly in the forms X. and P. The results already considered show empirically that μ cannot be determined by or correlated with any of these parameters. The evidence[3] indicates that in H' and H'' the shape constant for the rod part may be inherited independently of that for the cone part of the curve, and independently of changes in $F_{max.}$ or τ'. $F_{max.}$ may also be specifically heritable, or may show modifications. In the hybrids, τ' is increased. This could be formally explained as due to a lesser amount of excitable substance (or to a greater amount of an inhibitor) in all the elements of a set. Changes in $F_{max.}$ are clearly to be understood as due to alteration in the total number of elements of induced effect; this does not mean increased numbers of cells, but refers to the sort of increase in $F_{max.}$ (without change of $\sigma'_{\log I}$) which can be produced by shortening the light flash duration in the cycle—although this involves diminution of τ'. It would be of some interest in this connection to know whether μ would be the same if the percentage light time in the flash cycle were altered. We are more concerned, however, with the heritably invariant shape constants ($\sigma'_{\log I}$) of the F-log I contours and their independence of μ.

The designation of heritable attributes of organic constitution has been based fundamentally upon the recognition of rather gross differences between properties which behave as alternates in inheritance. The tangle of hypotheses and suppositions which has grown up about the attempt to systematize the analysis of less clear examples has added nothing of an independent character to the theory of the gene. From this standpoint a

gene is a label for the entity which in an operational sense exhibits a particular behavior in breeding experiments. The elaboration of the evidence that in the germinal chromosomes there is found a mechanism which parallels in minute detail the behavior required of the basis of segregation, linkage, crossing over, and spatial interrelationships of heritable units provides empirically a physical explanation of the assortment of the determinants of characteristics; but tells nothing as to the nature of their control. Active realization of the elementary fact that the individual manifestation of organic properties is achieved in the course of individual development has served only to sharpen the difficulty of theoretical genetics. On the one hand we have the numerical results of breeding tests in which developed properties are the markers which permit recognition of the distribution of heritable units; on the other hand we have the known behavior of chromosomal elements. The temptation has been insistent to account for the "nature" of a gene in terms of the physicochemical properties which the size and rationally presumed activities of the chromosomal gene require. The net result, as one finds it at the moment in the body of "physiological genetics," is fairly to be characterized as a mass of curiously unconvincing assumptions concerning the involvements of enzymes and unsatisfying analogies with the physicochemical manifestations of reaction rates.

Experiment has shown that there exist definite properties of individual organisms which are quantitatively reproducible, and heritable in a simple manner, but which could not be recognized at all by means of the customary and by now practically static and crystallized methods of genetic investigation.[33] The observable nature of several of these properties indicates the origin of at least a part of the prevalent confusion in genetic theory. Their investigation plainly suggests the kind of procedure which may aid materially in putting theoretical consideration of these matters upon a more productive foundation. The properties concerned are typically those characteristics of individual organisms which are to be termed capacities to exhibit particular modes of performance. A capacity for performance can be estimated only in terms of measured performance under defined conditions. It is easy to demonstrate in an elementary way that the index

[33] Crozier, W. J., and Pincus, G., 1929–30, *J. Gen. Physiol.*, **13**, 57. *Proc. 6th Internat. Cong. Gen.*, 1932, **2**, 31. *J. Gen. Physiol.*, 1936–37, **20**, 111. The essential point has in certain features been realized by Hogben and others, but not completely (*cf.* Hogben, L., Nature and nurture, 1933, New York, W. W. Norton & Co.; and Dickson, H., 1939, *Ann. Bot.*, **3**, N.S., 113.

of such a *capacity* can be obtained only by defining the relationship between measured performance and values of a controlled variable responsible for its manifestation. Consider the situation described by the curves in Fig. 1, and especially at the right hand side of this figure. These portions of the curves are reproduced in Fig. 4. Genetic analyses are usually, in fact almost exclusively, based upon the performance of individuals under constant (*i.e.*, the same) conditions. In the present instance this corresponds to testing the flicker responsiveness of *X.*, *P.*, and *H″* fishes, under fixed

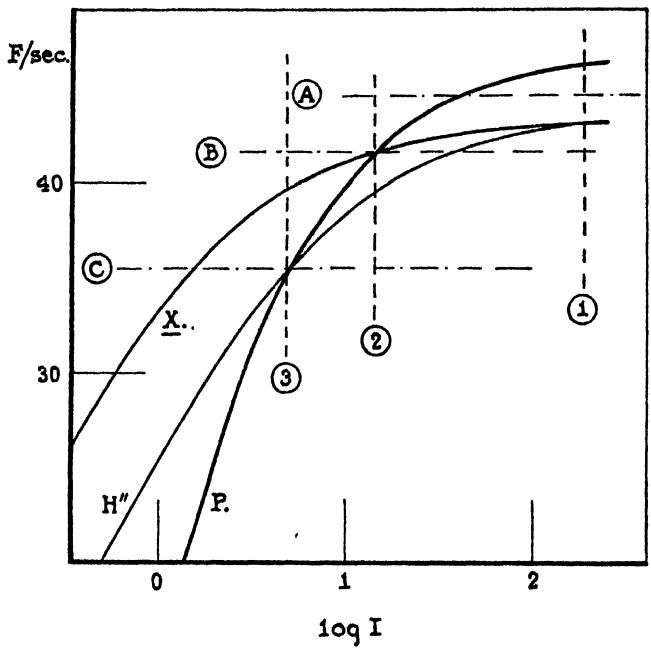

FIG. 4. The upper end of the graphs in Fig. 2, with dashed lines added to indicate the relationships which would appear if observations seeking to compare the responsiveness of *X.*, *P.*, and F_1 were to be made at constant conditions of *F* or of *I*.

conditions of temperature, of t_L/t_D, and of antecedent dark adaptation, and of either flash intensity or flash frequency (it being impracticable to measure the internal sensory disturbance at the threshold state for just detectable response when it is forced to appear). Since there is no *a priori* guide for the selection of any specific set of conditions we have a right to compare, on the basis of the information in Fig. 1, the results to be obtained by selecting in turn a number of different conditions under which the comparison is to be made. If these in fact lead to contradictory results we

must conclude that this general method of comparison is incapable of leading to significant deductions.

Suppose we had chosen the comparison implied by line *A* in Fig. 4. We would conclude that *P.* and *X.* clearly differ in the sense that *P.* is flicker responsive, *X.* not; and H'', their F_1 hybrids, would obviously be like *X.* If instead we had happened to choose as working environment the conditions signified by flash intensity level *1*, we would conclude that *X.* responds at a lower flash frequency than *P.*, and that the F_1 hybrids H'' are quantitatively like *X.*; at flash intensity *3*, however, if we had happened to select this, and had at our disposal only the results at this level, we would be compelled to consider that *X.* is typified by a higher critical *F* than *P.* and that H'' is precisely like *P.*; at position *2*, or *B*, *X.* and *P.* would be found indistinguishable but H'' either lower (in F_c) or higher (I_c) than either, depending on the criterion for elicitation of performance we chanced to be using. It is clear that only by acquaintance with the whole curve can we hope to institute comparisons between individuals which can be used for interpretative analysis. In this way alone can we avoid the confusion inescapable when arbitrarily fixed conditions circumscribe the exhibition of differences between individuals concerned in breeding experiments. And a large and significant factor of uncertainty exists in any given case of whatever kind when the invariance of the characteristics involved with respect to developmental factors has not been demonstrated. Apart from this, unless the nature of such dependence has been established, we are left with mere markers of the existence of functional differences. These markers, in general, give no information whatever as to the kinetic character of the processes from which they result.

In many cases the recognizable features of performance characteristics necessarily depend upon the integrated or at least collective actions of a number of cells or other units. The summated actions of these units inevitably exhibit properties involving the distribution of contributive capacities among the units present. That this distribution is one of uniformity is excessively unlikely. That it may be random or according to some other fixed rule is to be decided from the data. The problem then arises, how is it possible for an assemblage of units to provide summated effects following a simple law which is physically interpretable? A full discussion of this problem, would take us far from our immediate purpose, because the question is decidedly a fundamental one for many kinds of measurements with biological systems. It is insistent in connection with measurements of the relation between temperature and "velocity" of per-

formance by cell aggregates,—for example, respiration per unit time in cell suspensions; or photosynthetic liberation of O_2; or, in the present data, the velocities of processes governing photic excitation.

Such relationships, when dynamically simple so far as the properties of the measurements reveal, must be taken to signify that the same controlling process is at work in each of the elementary units concerned; the effects which it produces are randomly distributed, as if there were a random distribution of amounts of the governing catalyst.

It thus becomes a matter of very considerable importance to obtain instances in which there may be achieved a means of analytically separating

TABLE III

Mean critical intensities for response at various flash frequencies ($t_L = t_D$; 21.5°) for 17 F_2 individuals (three observations on each of the 17 at all points) produced by 1 F_1 ♂ and 2 F_1 ♀ ♀ from *X. montezuma* and *P. maculatus*. Log I_m (millilamberts) and log P.E.$_{1I_1}$ are not distinguishable from values for F_1 individuals (in Crozier, W. J., and Wolf, E., *J. Gen. Physiol.*, 1938–39, **22**, 463). See Fig. 4.

F per sec.	log I_m	log P.E.$_{1I_1}$
2	$\bar{6}.4440$	$\bar{8}.7989$
3	$\bar{6}.8193$	$\bar{7}.0022$
4	$\bar{5}.1235$	$\bar{7}.5355$
5	$\bar{5}.8298$	$\bar{6}.0777$
6	$\bar{4}.5441$	$\bar{6}.9659$
8	$\bar{3}.7317$	$\bar{5}.8985$
10	$\bar{2}.7330$	$\bar{3}.2541$
15	$\bar{1}.4043$	$\bar{3}.7954$
20	$\bar{1}.6772$	$\bar{3}.9831$
30	0.2615	$\bar{2}.7479$
40	1.2355	$\bar{1}.4629$

the purely statistical parameters from those giving opportunity for kinetic characterization. The present data supply an instance. Certain statistical parameters for rod and cone sections of the *X.* and *P.* curves (Fig. 1) are specific and determinate, as proved directly by the results of the breeding tests. That these parameters ($F_{max.}$ and $\sigma'_{\log I}$) *are* essentially statistical is proved by the manner in which they are (nonspecifically) dependent upon retinal area and light time fraction in the flash cycle.[34] The abscissa of inflection (τ'), however, is a simple function of temperature. The descriptive constant in this relationship (μ) is independent of $F_{max.}$ and of $\sigma'_{\log I}$,

[34] *J. Gen. Physiol.*, 1937–38, **21**, 223, 313, 463.

and is the same in H'' as in the parent stocks $X.$ and $P.$ Thus it must be concluded that, since $F_{max.}$ and $\sigma'_{\log I}$ can follow one parent precisely in the hybrid offspring, the purely statistical properties exhibited by performance

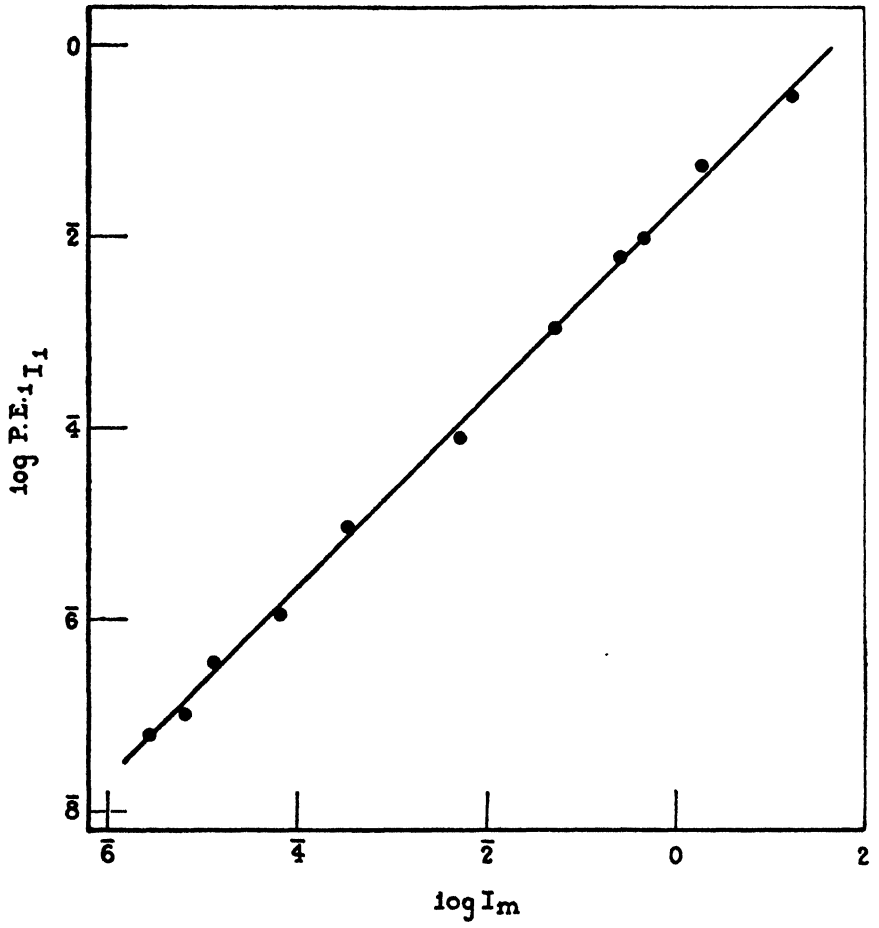

FIG. 5. The variation of I_1 is directly proportional to I_m in the data on F_2 individuals from $X. \times P.$, and the proportionality constant is the same as for F_1 (see text), $n = 3$, $N = 17$ (*i.e.*, fifty-one readings at each point).

capacity may be inherited without the expression of a chemical difference in the mechanism governing the performance.

The crossing of $X.$ and $P.$ undoubtedly brings into play conditions well known to be involved in interspecific and intergeneric crosses. Only certain genetic combinations are viable, and peculiar conditions arise in the sex manifestations, so that frequently such hybrids tend to breed true. Thus

in our earlier experiment[2] backcrosses of $F_1 \times X$. gave (H') animals providing a condition of the F-log I contour essentially like that of the present F_1 ($= H''$); the minor differences (of τ', and in the shape of the rod branch) could be explained by differences of species in the X. and P. parental stocks. So also, several batches of F_2 hybrids produced from 1 ♂ and 2 ♀ ♀ F_1's gave data (Table III) not to be distinguished quantitatively from those for F_1 at the same temperature, either in the values of I_m or of the inter-individual variability. Simple segregations are not necessarily to be looked for, in general, in such material. Color segregations were apparent in the expected way: of 17 individuals, 11 were "gray" and 6 "gold." What is important, rather, is the stability of the descriptive constants introduced as result of the crossing.[35]

VII

SUMMARY

For the teleosts *Xiphophorus montezuma*, *Platypoecilius maculatus*, and their F_1 hybrids the temperature characteristics (μ in Arrhenius' equation) are the same for the shift of the low intensity and the high intensity segments of the respective and different flicker response contours (critical intensity I as a function of flash frequency F, with light time fraction constant, at 50 per cent). The value of μ is 12,500 calories or a very little less, over the range 12.5 to 36°. This shows that $1/I$ can be understood as a measure of excitability, with F fixed, and that the excitability is governed by the velocity of a chemical process common to both the classes of elements represented in the duplex performance curve (rods and cones).

It is accordingly illegitimate to assume that the different shapes of the rod and cone branches of the curves are determined by differences in the chemical mechanisms of excitability. It is also forbidden to assume that the differing form constants for the homologous segments in the curves for two forms (X. and P.) are the reflections of a difference in the chemical factors of primary excitability. These differences are determined by statistical factors of the distribution of excitabilities among the elements implicated in the sensory effect *vs.* intensity function, and are independent of temperature and of the temperature characteristic.

[35] Genetic information about these fishes is referred to in summaries by Goodrich, H. B., *Quart. Rev. Biol.*, 1929, **4**, 82, and Goldschmidt, R., *Quart. Rev. Biol.*, 1937, **12**, 426. Koswig in particular has used "*X. montezuma*" and *P. maculatus* (*Z. indukt. Abstammungs- u. Vererbungsl.*, 1931, **59**, 61), but these observations have been concerned with sex and color factors.

It must be concluded that the physicochemical nature of the excitatory process cannot be deduced from the shape of the performance contour.

The form constants ($\sigma'_{\log I}$ and $F_{max.}$) for F *vs.* log I are specifically heritable in F_1, although μ is here the same as for X. and P. In an intergeneric cross one cannot in general expect Mendelian simplicity of segregation in subsequent generations, and in the present case we find that F_2 individuals are indistinguishable from F_1, both as regards F *vs.* log I and as regards the variation of I within a group of 17 individuals. The result in F_2 definitely shows, however, that certain specific statistical form constants for the F-log I contour are transmissible in inheritance. It is pointed out that there thus is provided an instance in which statistical (distribution) factors in performance characteristics involving the summating properties of assemblages of cellular units are heritable in a simple manner without the implication of detectable differences in chemical organization of the units involved. This has an important bearing upon the logic of the theory of the gene.

THE CHEMISTRY OF HUMAN SKIN

IV. The Electrokinetic Effect of Various Ions upon Suspended Particles of Stratum Corneum

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The conditions which control the transmission of fluids across the human epithelium are important in connection with the local introduction of soluble substances. In 1924 Rein (1) investigating human skin by electroendosmosis concluded that the rate of flow toward the cathode was greater in distilled water than in any neutral salt solution. The inhibition increased with increased concentration and the specific effect of the cations was according to the series Al > Ca > Na > K.

For some time it has been known that under a given potential difference a simple relationship may exist between the velocity of a particle relative to the liquid in which it is suspended and the volume of flow of the liquid streaming through a membrane having the same chemical constitution as the surface of the particle. Both the velocity of migration and the volume of flow are proportional to the electrokinetic potential existing across the effective double layer. This is particularly true for protein surfaces (2–3) and one would expect the results obtained by electrophoresis to be comparable to those reported by electroendosmosis.

EXPERIMENTAL

The stratum corneum of the human skin was washed with water, extracted with acetone, alcohol, and ether; dried in a vacuum at body temperature and powdered in a ball mill. The particles were suspended in 0.1 M to 0.0001 M solutions of the chlorides, bromides, and iodides of lithium, sodium, potassium, and rubidium and the chlorides of calcium, barium, and aluminum.

The Northrop-Kunitz microelectrophoresis cell was employed as before (4) for measuring V, the electrical mobility in μ per second per volt per cm.

The electrokinetic potential was calculated from the equation (5)

$$\zeta = \frac{4\pi\eta_0 V}{DX}$$

where η_0, D, and X represent respectively the coefficient of viscosity, the dielectric constant, and the field strength of the medium. The assumption was made that the values of D and η_0 as calculated for the bulk of the liquid remained unchanged in the effective double layer.

The electrical charge per unit area which gives rise to the electrokinetic potential at the surface was determined from the relationship expressing σ, the surface charge density as a function of ζ, the potential (6)

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}} \sqrt{\Sigma c_i \left(e^{-z_i \frac{e\zeta}{KT}} - 1\right) + \Sigma c_j \left(e^{+z_j \frac{e\zeta}{KT}} - 1\right)}$$

where N is Avogadro's number, D, the dielectric constant, K, Boltzmann's constant, T, the absolute temperature, c_i and c_j, the concentration of the cations and anions in mols per liter, z_i and z_j, the valences of the cations and anions and σ has the same sign as ζ.

DISCUSSION

The results are expressed by curves which appear in Figs. 1, 2, 3, and 4.

Fig. 1 shows that the skin whose isoelectric point is pH 3.7 (4) assumes, as would be expected, a negative charge in distilled water. In solutions of neutral uni-univalent salts (see also Figs. 2 and 3) the electrokinetic potentials, which depend upon the charge of the particle and the thickness of its double layer, plotted against concentration produce curves with well defined maxima that are more characteristic of inert substances than for protein materials (7). Usually the charge on protein surfaces is determined by the hydrogen ion activity and dilute electrolytes influence the charging process only in so far as they are able to produce a change in the dissociation of H and OH ions. On inert surfaces the charge is quite sensitive to small changes in electrolytic concentration, increasing with increased concentration until a limiting value is reached and the mechanism involved appears to be tantamount to preferential adsorption. Under both circumstances increasing salt concentrations ultimately decrease the ζ-potential by diminishing the thickness of the effective double layer. The ζ-C curves produced by the skin particles in neutral solutions suggest that in addition to the charge incident to the pH, a charging process similar to that which occurs for inert surfaces obtains.

The maxima are less defined for the di-univalent compounds while for corresponding concentrations of the tri-univalent salt there is a reduction of the electrokinetic potential to zero and subsequent sign reversal. This is the usual effect of polyvalent ions of opposite sign upon protein surfaces and is probably, in this instance, due to the combined result of the prefer-

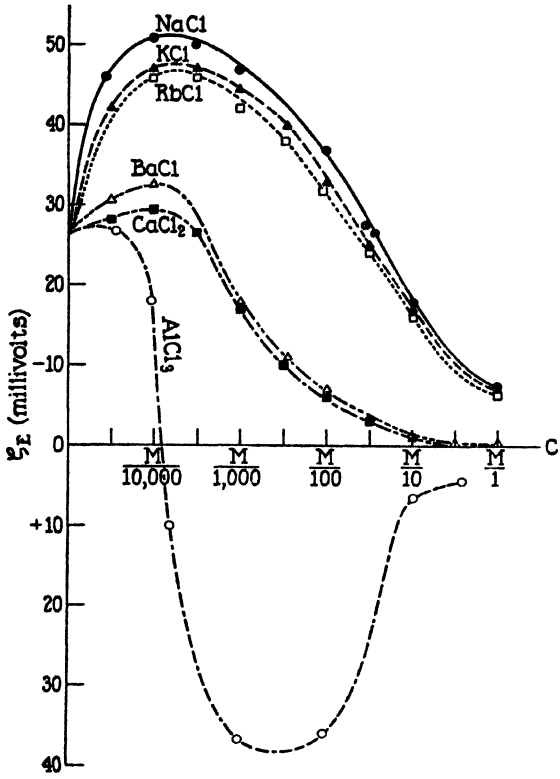

FIG. 1. The effect of salts on the ζ-potential of skin

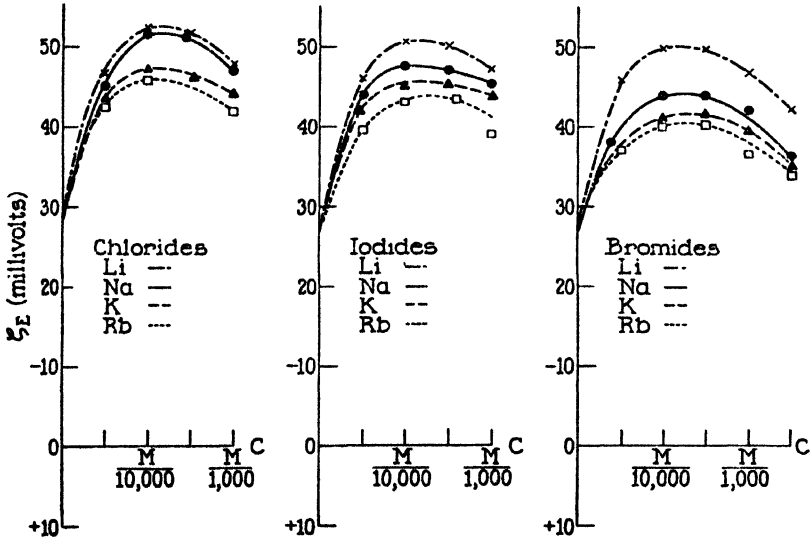

FIG. 2. Alkali halides with common negative ions

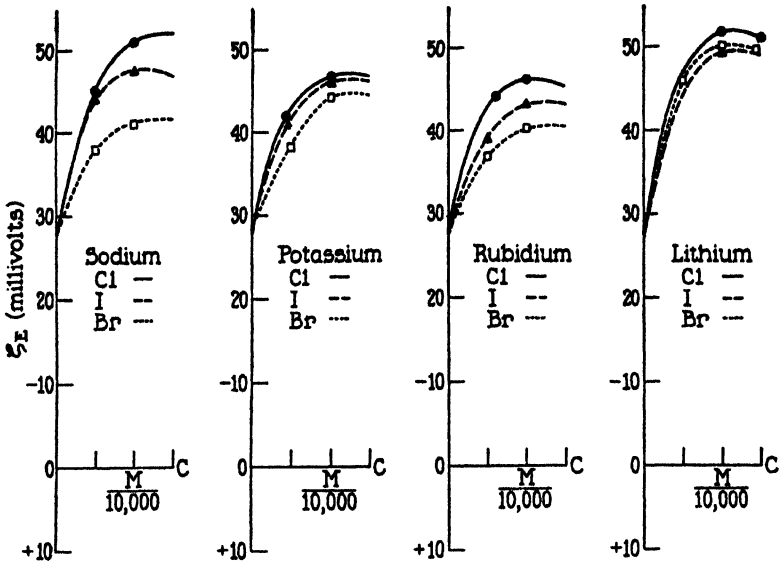

FIG. 3. Alkali halides with common positive ions

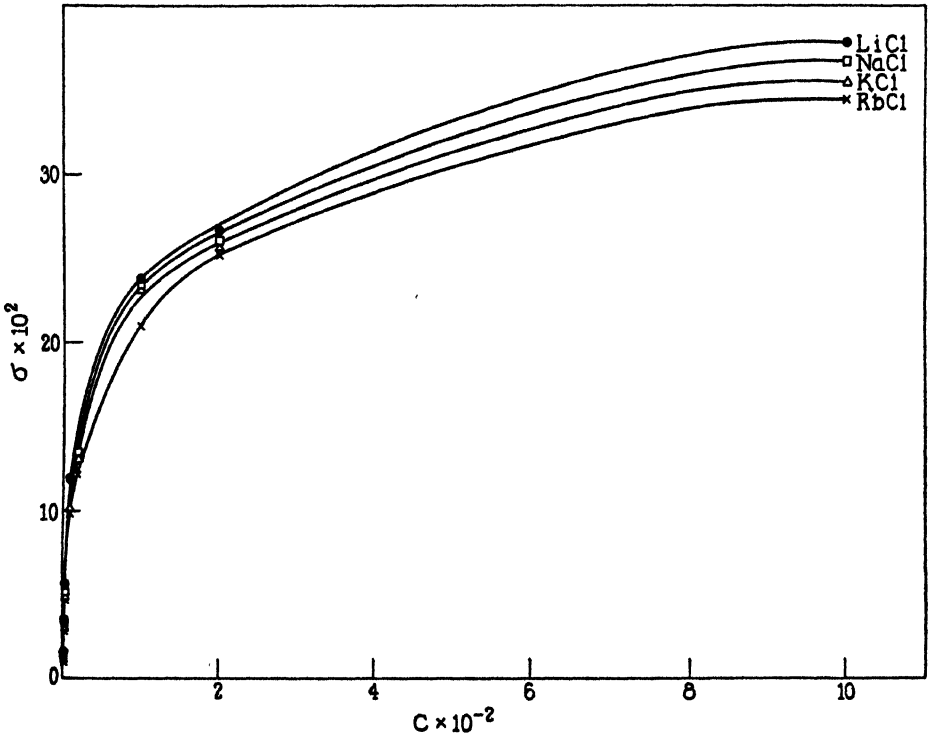

FIG. 4. The electric charge density of the skin surface in various salt solutions

ential adsorption of the cation and the change in hydrogen ion concentration incident to the hydrolysis of the aluminum chloride.

The inhibitory effect of the cations is represented by the series Al > Ca > Ba > K > Na, which is essentially the same order as that obtained by electroendosmosis (1). The conclusions by Rein that the endosmotic flow is greatest in distilled water and is diminished by the addition of salts can only be justified by considering the concentrations used. Had more dilute solutions been employed and the material freed from tissue fluids the indications are that the rate of flow would have been greatest for the uni-univalent salts in concentrations between 0.0001 M and 0.0002 M, inasmuch as it follows from theoretical considerations that any increase in the electrokinetic potential will result in a corresponding increase in electroendosmotic flow. The aluminum salt in reversing the sign of the potential produces a point of no flow between the charge assumed in distilled water and the maximum positive potential. The alkali halides with common negative ions (Fig. 2) affect the electrokinetic potential in the order Li > Na > K > Rb. The apparent ionic specificity is not peculiar to skin alone since the same order is shown on such inert interfaces as cellulose and glass (8–9). Similar salts with common positive ions exhibit the series Cl > I > Br (Fig. 3) in contrast to the order I > Br > Cl frequently observed on passive interfaces (10). This seems to indicate a selective adsorption of chloride ions upon the skin surfaces which is coincident with the observation that the concentration of chlorides per unit of water is normally high in this tissue (11).

The charge as well as the potential must be considered in any attempt to evaluate surface chemical phenomena. A rather simple curve is obtained when the electric charge density is plotted against concentration. On addition of traces of salts not reversing the sign σ increases rapidly reaching a limiting value at approximately 0.01 M (Fig. 4). The course is not characteristic since it is given by a variety of inert substances and appears to be independent of the ion or the nature of the surface (10).

SUMMARY

1. The electrophoretic activity of particles of human skin in distilled water and different concentrations of salt solutions has been studied. The electrokinetic potential and the charge density were determined and comparisons made with results obtained by electroendosmosis.

2. The electrokinetic potential is ultimately decreased if sufficient salt is added. The order of inhibition is Al > Ca > Ba > K > Na.

3. The lyotrophic series Li>Na>K>Rb and Cl>I>Br express respectively the comparative effect of the monovalent cations and anions upon the electrokinetic potential of the skin.

4. Since both electrophoresis and electroendosmosis are dependent upon the electrokinetic potential, it follows from the results obtained, that the greatest rate of flow through the human skin under the force of an applied electrical current would be given by concentrations of neutral salts between 0.0001 M and 0.0002 M.

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CALCULATIONS OF BIOELECTRIC POTENTIALS

VI. Some Effects of Guaiacol on Nitella

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Important changes are produced in the apparent mobilities and partition coefficients of inorganic ions by applying guaiacol to *Nitella*. The calculations indicate that guaiacol may increase apparent mobilities 3-fold and partition coefficients more than 50-fold. These effects are completely reversible.[1]

The apparent mobilities were calculated, as explained elsewhere,[2] from the P.D. between 0.01 M and 0.001 M for each salt. The results[3] are given in Table I (column 3).

The order of mobilities of the alkali metals follows that in water until we reach cesium.[4] In water the mobility of Cs^+ exceeds that of K^+ but in *Nitella* it is much less. This is also true of *Valonia*[5] and of *Halicystis*.[6] In these cases Cs^+ acts like Na^+. This seems to be the case also with

[1] The experiments were made on *Nitella flexilis*, Ag., at 20–25°C., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). The cells had the same treatment as described in the previous paper on the effects of guaiacol (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417). The concentration of guaiacol was in all cases 0.02 M.

Like those discussed in the previous paper, these cells belonged to Lot B. There was no evidence of injury in these experiments.

The RbCl and CsCl were obtained from Theodor Schuchardt. The remaining salts were obtained from Kahlbaum.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417. In all cases the change was made from the dilute to the concentrated solution and *vice versa:* in the former case the effect of action currents must be taken into account.

[3] All mobilities are calculated by putting the mobility of Cl, or v_{Cl}, equal to unity. Calculated on this basis the mobilities in water at 25°C. are as follows: Li 0.5, Na 0.65, NH_4 0.96, K 0.96, Rb 1.03, Cs 1.04, ½ Mg 0.7, ½ Ca 0.78, Cl 1.

[4] The behavior of cesium is exceptional in respect to permeability in *Valonia* where its rate of entrance is exceedingly slow (*cf.* Cooper, W. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928–29, **12**, 427).

[5] *Cf.* Damon, E. B., *J. Gen. Physiol.*, 1938–39, **22**, 819.

[6] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 53.

muscle and nerve. If we arrange the ions in the order of their effect in producing negativity in *Nitella* we have K, Rb > Na > Li > Cs (Table I, column 6). This does not differ much from the series found in *Valonia*[7] and in *Halicystis*,[6] as well as in muscle and nerve.

According to Höber[8] the order in frog muscle is K > Rb > NH_4 > Cs > Mg > Na > Li. For the sciatic nerve of the frog[9] we have K > Rb > NH_4 > Cs > Na, Li. For the nerve of the spider crab[10] we have K > Rb > Cs.

It is a striking fact that these cells react so differently to pairs of ions, *e.g.* Na^+ and K^+, K^+ and Cs^+, Rb^+ and Cs^+, which are chemically similar.[11] This deserves further study.

Of especial interest is the increase in apparent mobilities produced by guaiacol (Table I, next to the last column). As an example we may take u_{Na} which is raised from 2.33 to 7.30 by the action of guaiacol. The P.D. between 0.01 M and 0.001 M NaCl increases from 23.2 to 44.0 when guaiacol is applied.

The question arises whether this is because the protoplasmic surface is acting more like a layer of guaiacol. To answer this some guaiacol (previously shaken with 0.001 M NaCl) was placed in a U-tube with 0.001 M NaCl at one side and 0.01 M NaCl at the other.[12] On leading off from the aqueous solutions to a Compton electrometer we observed a potential of about 10 mv. (dilute solution positive in the external circuit). Hence if the protoplasmic surface acted like guaiacol we should not expect a concentration effect of 44 mv. With KCl the concentration effect with guaiacol was about 15 mv.

The chemical effect, *e.g.* the P.D. between 0.01 M KCl and 0.01 M NaCl presents a different picture. This chemical effect in *Nitella* amounts to 94 mv. which is reduced to 20.9 by the application of guaiacol. With guaiacol in the U-tube the corresponding value is about 14 mv. Hence

[7] Damon, E. B., *J. Gen. Physiol.*, 1938–39, **22**, 819. In *Valonia*, u_{Cs} like u_{Na} is less than v_{Cl}.

[8] Höber, R., *Arch. ges. Physiol.*, 1905, **106**, 599. See also Seo, T., *Arch. ges. Physiol.*, 1924, **206**, 485.

[9] Netter, H., *Arch. ges. Physiol.*, 1928, **218**, 310. See also Wilbrandt, W., *J. Gen. Physiol.*, 1936–37, **20**, 519.

[10] Cowan, S. L., *Proc. Roy. Soc. London, Series B*, 1934, **115**, 216. See also Wilbrandt, W., *J. Gen. Physiol.*, 1936–37, **20**, 519.

[11] The chemical similarity is greater in the case of K^+ and Na^+ than in the other pairs.

[12] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417.

in this respect the protoplasmic surface acts somewhat more like guaiacol after guaiacol is applied.

It is not surprising that we are unable to predict the effects of guaiacol in the protoplasmic surface which is undoubtedly a mixture of substances concerning which our knowledge is very limited. As might be expected, guaiacol does not affect all cells in the same way. We find that *Nitella* resembles *Halicystis*[13] in that the mobility of Na^+ is increased by guaiacol but that of K^+ is not affected. In *Valonia*[14] the mobility of Na^+ is increased and that of K^+ is decreased.

In addition to the alkali metals mentioned, NH_4^+, Mg^{++}, and Ca^{++} were employed. Regarding NH_4^+ it may be said that its mobility is only about half that of K^+ although in water the two mobilities are nearly equal. We see that the mobility of ½ Mg^{++} and that of ½ Ca^{++} are greater than that of K^+ which is not the case in water. It seems probable that Mg^{++} and Ca^{++} do not obey very well the equations here used since it is possible that they produce alterations in the surface.

Let us now consider the partition coefficient S (S = concentration in the non-aqueous protoplasmic surface layer ÷ concentration in the external solution). This was determined as previously explained[12] by measuring the P.D. of 0.01 M KCl against 0.01 M of each salt in turn. The partition coefficient was calculated as follows. In the case of NaCl, for example, we find by trial what value of $S_{NaCl} \div S_{KCl}$ will give the observed P.D. of 44 mv. employing the mobilities already found; *i.e.*, $u_K = 8.76$ and $u_{Na} = 2.33$. We thus obtain $S_{NaCl} \div S_{KCl} = 0.0263$. This is done for each salt in turn. The results are shown in Table I.

We can compare the partition coefficients before and after the application of guaiacol since, as shown in a previous paper,[12] the partition coefficient of KCl is not changed by guaiacol.[15] The partition coefficients of the alkali metals increase as the ionic radius increases until we come to cesium which is exceptional. This increase is in line with the suggestions of Shedlovsky and Uhlig.[16]

The application of guaiacol raises all the partition coefficients except those of KCl and RbCl (Table I, last column). Too little is known about the theory of partition coefficients to make it desirable to comment on this

[13] Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 707.

[14] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

[15] To calculate the partition coefficient in the presence of guaiacol we employ the mobilities found in the presence of guaiacol, *e.g.* $u_{Na} = 7.30$ instead of 2.33. The value of u_K is not changed by guaiacol (see footnote 12).

[16] Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933–34, **17**, 563.

but it may be stated that the value of S for guaiacol in contact with aqueous solutions of NaCl and KCl is very low[17] (about 0.001).

According to the calculations the partition coefficient of $MgCl_2$ is very low. This might be expected on chemical as well as on biological grounds. We might expect that of $CaCl_2$ to be equally low: this is not the case.

TABLE I

*P.D.'s and Partition Coefficients of Chlorides (MCl or MCl_2) with and without Guaiacol**

MCl or MCl_2	Concentration effect† 0.01 M vs. 0.001 M				Chemical effect‡ MCl or MCl_2 0.01 M vs. KCl 0.01 M				Value with guaiacol / Value without guaiacol	
	No guaiacol		With 0.02 M guaiacol		No guaiacol		With 0.02 M guaiacol			
	P.D.	u_M	P.D.	u_M	P.D.	$\frac{S_M}{S_K}$	P.D.	$\frac{S_M}{S_K}$	u_M	$\frac{S_M}{S_K}$ §
LiCl ...	19.8	2.04	41.8	5.83	108.8	0.0134	48.6	0.13	2.86	9.7
NaCl	23.2‖	2.33	44.0‖	7.30	94.0	0.0263	20.9	0.426	3.13	16.2
KCl¶.....	46.1‖	8.76	46.1‖	8.76	zero	1.0	zero	1.0	1.0	1.0
RbCl¶.....	46.0	8.76	46.0	8.76	zero	1.0	zero	1.0	1.0	1.0
CsCl.	15.3	1.72	31.5	3.38	113.1	0.0132	23.7	0.82	1.97	62.1
NH_4Cl.. .	35.0	4.05	38.4	4.91	94.7	0.0182	13.8	0.935	1.23	51.4
$MgCl_2$¶**. .	25.0	20.7	25.0	20.7	112.0	0.0017	43.6	0.048	1.0	28.2
$CaCl_2$**.....	18.8	7.52	23.4	14.46	94.9	0.02	25.8	0.323	1 92	16.1

* The number of observations was usually 8 or 10 and the probable error of the mean less than 7 per cent of the mean.

† The dilute solution is in all cases positive in the external circuit.

‡ KCl is in all cases negative in the external circuit.

§ S_K is the same with and without guaiacol.

‖ Values taken from Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417.

¶ The P.D.'s for the concentration effect were the same within experimental error.

** Here u_M refers to ½ Mg or ½ Ca.

To what extent are these calculations valid? One way of testing this is to try to predict the P.D.'s of various dilutions, *e.g.* of KCl and NaCl, from the calculated values of u_{Na}, u_K, S_{NaCl}, and S_{KCl}. This has been done with satisfactory results[18] with NaCl, KCl, and NH_4Cl.

We may also try to predict chemical effects. For example, in the present paper, using the values (in absence of guaiacol) for the mobilities calculated from the concentration effects and for the partition coefficients calculated

[17] For guaiacolates it is higher (*cf.* Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 469). It is higher for salicylates than for chlorides.

[18] In unpublished work. For published data see Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

from the observed P.D.'s of 0.01 M NaCl *vs.* 0.01 M KCl and of 0.01 M LiCl *vs.* 0.01 M KCl we may predict the P.D. of 0.01 M LiCl *vs.* 0.01 M NaCl. The prediction is 8.7 mv. and the observed value is 8.0 mv. (NaCl negative to LiCl in the external circuit). For 0.01 M RbCl *vs.* 0.01 M NaCl the prediction is 94 mv. and the observed value 102 (RbCl negative). For 0.01 M CsCl *vs.* 0.01 M NaCl the prediction is 11.0 mv. and the observed value is 12.6 mv. (NaCl negative). When we are not dealing with alkali metals the prediction is somewhat less satisfactory.

The success of these predictions for the alkali metals and the fact that the mobilities and partition coefficients of the alkali metals as calculated in the present paper are reasonably in line with expectation indicate that the method of calculation may be trusted to a certain extent. We must be on our guard, however, against secondary effects.[19]

If concentrations are too high or exposures too long such secondary effects may appear: such effects are, of course, not predicted by these equations. These secondary effects may be reversible up to a certain point beyond which they become irreversible. They doubtless involve structural alterations,[20] and may be brought about by non-electrolytes as well as by electrolytes.

We may therefore speak of primary effects and secondary effects. By primary effects we mean those which involve no structural alteration of the protoplasmic surface. If, for example, the potential of the protoplasm is largely due to an outwardly directed concentration gradient of potassium[21] we abolish the potential when we abolish the concentration gradient: this we do by placing the proper concentration of potassium outside.[22] Here there is no need to assume any structural alteration of the surface.

A change in the chemical composition of the surface might conceivably occur without any structural change. Possibly guaiacol produces this kind of alteration.

[19] Such secondary effects are much more apt to occur with alkaline earths than with alkali metals. For a striking effect of calcium see Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 223; Blinks, L. R., Rhodes, R. D., and McCallum, G. A., *Proc. Nat. Acad. Sc.*, 1935, **21**, 123.

It may be mentioned in this connection that the concentration effect of 0.01 N KCl + 0.01 N $CaCl_2$ *vs.* 0.001 N KCl + 0.001 N $CaCl_2$ is decreased by guaiacol, contrary to the result obtained with either salt singly.

[20] *Cf.* Höber, R., Andersh, M., Höber, J., and Nebel, B., *J. Cell. and Comp. Physiol.*, 1939, **13**, 195.

[21] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, [illegible]9; *J. Gen. Physiol.*, 1934–35, **18**, 215.

[22] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 391.

The fact that both primary and secondary effects may be inhibited to a certain extent by alkaline earths[23] does not, of course, mean that they are identical. It may signify that alkaline earths decrease the solubility in the protoplasmic surface of the various substances which depress the potential.[24] This view is favored by the fact that it requires a very high concentration of alkaline earths to be effective and in *Nitella* they fail to inhibit the depressing action of potassium when its concentration is raised to 0.1 M.[24]

SUMMARY

Values have been calculated for apparent mobilities and partition coefficients in the outer non-aqueous layer of the protoplasm of *Nitella*. Among the alkali metals (with the exception of cesium) the order of mobilities resembles that in water and the partition coefficients (except for cesium) follow the rule of Shedlovsky and Uhlig, according to which the partition coefficient increases with the ionic radius.

Taking the mobility of the chloride ion as unity, we obtain the following: lithium 2.04, sodium 2.33, potassium 8.76, rubidium 8.76, cesium 1.72, ammonium 4.05, ½ magnesium 20.7, and ½ calcium 7.52.

After exposure to guaiacol these values become: lithium 5.83, sodium 7.30, potassium 8.76, rubidium 8.76, cesium 3.38, ammonium 4.91, ½ magnesium 20.7, and ½ calcium 14.46.

The partition coefficients of the chlorides are as follows, when that of potassium chloride is taken as unity: lithium 0.0133, sodium 0.0263, rubidium 1.0, cesium 0.0152, ammonium 0.0182, magnesium 0.0017, and calcium 0.02.

These are raised by guaiacol to the following: lithium 0.149, sodium 0.426, rubidium 1.0, cesium 0.82, ammonium 0.935, magnesium 0.0263, and calcium 0.323 (that of potassium is not changed).

The effect of guaiacol on the mobilities of the sodium and potassium ions resembles that seen in *Halicystis* but differs from that found in *Valonia* where guaiacol increases the mobility of the sodium ion but decreases that of the potassium ion.

[23] Höber, R., *Arch. ges. Physiol.*, 1905, **106**, 599. See also Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

[24] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

PERFORMANCE OF THE HEPP MICRO-OSMOMETER

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Experience with several types of osmometer for the measurement of "colloid" osmotic pressure of serum and similar fluids has led us to the conclusion that an apparatus based upon the principles of Van Campen (1) but adapted to volumes of about 1 cc. would be nearly ideal for the purpose. Such an apparatus appears to have been designed by Hepp (2). That the apparatus yields correct values was not demonstrated by him. The importance of such demonstration is evident from the recent paper of Zozaya (3) in which it is inferred that practically all published values for the colloid osmotic pressure of human serum are about 100 per cent too high.

The measurements tabulated below show that the instrument is accurate as well as precise, rapid, and convenient in use. To them we have added amplification of Hepp's description of certain aspects of its assembly and operation.

Accuracy

The instrument was standardized with a solution of horse serum albumin given us by Dr. T. F. McMeekin, Department of Physical Chemistry, Harvard Medical School. In water solution, the once crystallized and dialyzed preparation gave no color with Nessler's reagent and contained 0.4 per cent carbohydrate by the orcein test, an amount corresponding to 4 per cent globulin. To the stock aqueous solution were added the calculated amounts of KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ to produce a solution $\frac{0.8}{15}$ M $Na_2HPO_4 + \frac{0.2}{15}$ M KH_2PO_4, pH 7.4. The albumin content of the stock was 8.27 gm. per 100 cc. solution as determined by micro-Kjeldahl. The stock solution was diluted with phosphate buffer $\frac{0.8}{15}$ M $Na_2HPO_4 + \frac{0.2}{15}$ M KH_2PO_4 (pH 7.4 by phenol red), and the albumin concentration of each dilution estimated from duplicate micro-Kjeldahl determinations, the N

content of the albumin being taken as 15.8 per cent (McMeekin). The stock solution was kept at approximately 4°C. in a bottle containing also a test tube partly filled with toluol. Dilutions of the stock, similarly kept, appeared to be stable for at least 2 weeks.

The osmotic pressure of the diluted albumin solution was determined usually against its own ultrafiltrate, produced in the osmometer in about 20 minutes. The free membrane area utilized was usually 45 mm. in diameter. Nearly all measurements were made with one of Hepp's instruments, slightly modified from that described in his 1936 paper; the modification consists in utilizing suction on the capillary throughout the entire procedure instead of suction to fill the capillary followed by pressure on the protein solution when the final measurement is made.[1] All measurements were made at 25 ± 0.5°C.

From the observed osmotic pressures, the molecular weight of the horse serum albumin was computed. The accepted mean value is 72,000 ± 3,000 (Adair and Robinson (4); Burk (5)).

Two modes of computation were used (Adair and Robinson (4)). In the first, the ratio c/p (c = albumin concentration in grams per 100 cc. of solution, and p = observed osmotic pressure in millimeters of water) was plotted against c for $c = 0.713$ to $c = 5.12$; the plot, which was to the same scale as Adair and Robinson's Fig. 1, yielded a straight line. Extrapolation of the line to $c = 0$ gave c/p at infinite dilution equal to 0.0290. The molecular weight of the albumin preparation was then calculated from $M = 10\ RT\ [c/p]$, where $RT = (22.414)\ (760)\ (13.6)\ (298/273)$ and $[c/p] = c/p$ at $c = 0$. Hence $M = 73{,}400$.

The second method of computation permits the calculation of a value of M for each pair of values of p and c. This is possible by utilization of the empirical coefficient ϕ which represents the sum of the osmotic effects due to the ion pressure difference, volume of the protein hydrate, and deviations from the laws of ideal solutions in the formula $M = 10\phi\ RT\ c/p$. Values of ϕ based upon numerous determinations on several albumin preparations of c and p at 0° may be calculated from $\phi = \frac{1}{1 - Kc}$, where $K = 0.064$ (Adair and Robinson (4)). Utilization of these values of ϕ presupposes that M is constant at $c = 0.255$ to 5.12 and that our pairs of values of c/p and c may be considered part of the population of values from which Fig. 1 of

[1] An instrument similar to this modification of Hepp's original device was made for one of us (G. S.) in the machine shop of the Harvard Medical School and functions as well.

Adair and Robinson's (4) paper is drawn. The values of c, p, c/p, ϕ, and M for our sample of albumin are shown in Table I.

TABLE I

*Computation of M from Observed Osmotic Pressure**

c	p	c/p	ϕ	M
gm./100 cc. solution	*mm. H_2O*			
0.255	10		1.015	65,500
	8			81,800
0.267	11		1.02	62,700
	9			76,600
0.510	21		1.03	63,300
	20			67,200
0.534	19		1.04	73,600
	18			77,600
0.713	26	0.0280	1.05	72,800
	26	0.0280		72,800
1.02	37	0.0276	1.07	74,500
	38	0.0269		72,500
	37	0.0276		74,500
	39	0.0265		70,600
1.07	41	0.0261	1.07	70,600
	41	0.0261		70,600
	41	0.0261		70,600†
	41	0.0261		70,600‡
	41	0.0261		70,600‡
1.97	79	0.0249	1.15	72,500
	80	0.0248		71,600
3.12	139	0.0224	1.25	70,700
	137	0.0227		71,800
4.07	202	0.0202	1.35	68,800
	201	0.0203		69,200
	205	0.0199		67,800‡
	194	0.0210		71,700‡
5.12	271	0.0189	1.49	71,300†
	268	0.0192		72,000†
	272	0.0189		71,000‡

* All solutions measured against their own ultrafiltrates and with membrane area 45 mm. in diameter, except as noted otherwise.

† Phosphate buffer in capillary.

‡ Phosphate buffer in capillary and membrane area 30 mm. in diameter.

From Table I we may draw the following conclusions:

1. The Hepp osmometer yields as accurate results down to $c = 0.713$ gm. per 100 cc. as the standard instrument of Adair and Robinson (4). At smaller values of c, the scatter in value of M increases noticeably. This

is not surprising since a correction for capillarity has to be made. We have estimated the capillarity at the end of each measurement by taking the average of the height to which the column rises and to which it falls in the capillary. Values so obtained ranged from 21 to 23 mm. H_2O for albumin solution ultrafiltrates, and from 24 to 27 mm. H_2O for phosphate buffer in the capillary. Capillarity corrections so measured are of course not entirely satisfactory and constitute the chief absolute uncertainty of the method. Accuracy and precision of the instrument at pressures less than 25 mm. H_2O could be attained by attaching to the capillary a U-tube capillary (0.2 mm.) manometer, by means of a ground joint.

2. The values obtained are the same whether the capillary contains true ultrafiltrate or phosphate buffer. Hepp (2) showed that the capillary could contain distilled water, Ringer's, or 4× Ringer's as well as true ultrafiltrate in measurements of osmotic pressure of serum.

3. The same values are obtained with the 30 mm. diameter membrane (in which case 0.7 cc. gives a layer 1 mm. deep) as with the 45 mm. diameter membrane (in which case approximately 1.5 cc. are required to give a layer 1 mm. deep).

Attainment of Equilibrium

The speed with which equilibrium is approached is shown by the following experiment. With 0.9 per cent NaCl in the capillary and the total suction applied to the capillary about 130 mm. H_2O, a sample of approximately 6 per cent acacia in 0.9 per cent NaCl was placed in the osmometer. Readings were taken of the suction necessary to keep the meniscus in the capillary at a given mark on the ocular micrometer scale (magnification 60×). The readings are given in mm. H_2O after the times in minutes: 2, 136; 4, 142; 6, 155; 8, 172; 10, 188; 12, 200; 15, 215; 20, 232; 25, 242; 30, 250; 35, 258; 115, 261. Other samples of the same lot of acacia in NaCl (lot 3) had a colloid osmotic pressure of 250 to 260 mm. H_2O at 20°C. when measurements were made in the Krogh osmometer (cellophane No. 600 membranes) over periods of 24 to 72 hours (6).

The manometer readings often become steady within 5 to 15 minutes, if true ultrafiltrate is used and if it is possible (as when doing a determination in duplicate) to apply to the capillary column a suction approximately equal to the expected osmotic pressure before the protein solution is placed in the osmometer. Steady readings remain so for at least 4 hours.

A practicable time schedule of measurements for determinations in duplicate would then be 20, 30, and 60 minutes for the first sample; and

20 and 40 minutes for the second. Should the readings not be steady, additional ones would have to be made at 15 or 20 minute intervals. Most of the data recorded in Table I are based on measurements made at 20, 30, 45, 60, and 90 minutes.

Membrane Material

It is possible to use cellophane No. 450 and No. 600 in this type of osmometer but these materials are not satisfactory. They are so dense that a 0.2 mm. capillary with a capillarity correction of the order of 90 mm. H_2O must be employed. They are so thin that difficulty in securing a perfectly flat membrane is not infrequent. Finally, they are not necessarily uniform in properties and may give entirely inaccurate results. For example, Wies and Peters (7) report a large series of measurements on diluted and undiluted human sera with the Krogh osmometer; in these, all of the No. 600 cellophane discs used were cut out of a single sheet 1 meter square. The data show that in a number of instances a serum could be diluted 50 per cent while its osmotic pressure fell only 10 to 14 per cent or not at all; yet all determinations were made in duplicate.

We have tried reducing the density of cellophane by swelling cellophane No. 300 in 64 per cent zinc chloride as suggested by McBain and Stuewer (8), but could not produce membranes free of large wrinkles.

Collodion membranes suitable for protein concentration work were entirely too slow.

Elford membranes of average pore diameter 6 mμ were tried. They give the same results as the Zsigmondy filters but can be used only a few times.

Entirely suitable membranes are the Zsigmondy cellulose filters.[2] We have used the "ultrafine medium" and "ultrafine fine" filters as 6 cm. discs. The average life of these membranes with frequent use (four to eight measurements a day) at room temperature under pressures of the order of 30 cm. H_2O is 2 months. They keep well in 1 per cent HCl or in distilled water, unbuffered Ringer's, or NaCl solution to which a few crystals of thymol or 1 cc. formalin per 100 cc. solution have been added. They may be removed from the osmometer to one of these solutions between runs. It is advisable to use the membrane same side up all the time. Care should be taken that a membrane used with M/15 phosphate solutions is adequately washed with distilled water before use with calcium-containing solutions, for deposits of calcium phosphate render the membrane erratic and are difficult to remove completely with dilute HCl.

[2] Obtainable from Pfaltz and Bauer, Inc., Empire State Building, New York City.

Testing the Membrane

The ultrafine fine filters can be purchased protein-tight. The "medium" filters, which are appreciably faster and which were used for all the measurements in Table I, can be purchased tested for impermeability to benzopurpurin. In any case, a given filter can be examined for protein-tightness in the osmometer by ultrafiltering some serum or serum albumin solution into the capillary and carrying out a Heller's or similar test on the capillary contents.

For standardization of the membrane a calculation of the molecular weight of a satisfactory preparation of horse serum albumin from the observed osmotic pressure of a single solution having c equal to any value from 1 to 5 gm. per 100 cc. would seem satisfactory. The values of ϕ based upon Adair and Robinson's (4) measurements at 0°C. are evidently applicable to room temperatures; hence it appears unnecessary to determine M by the extrapolation method. Commercial samples of horse serum albumin appear not to be available.

Our experience is that it is unsafe to use any membrane which has not been standardized, and that standardization is again desirable whenever the behavior of a given membrane appears to be erratic. Occasionally membranes are unusable when received.

Apparatus Parts[3]

The glass plate we have used is 6 cm. in diameter and 7½ mm. thick; it is bored centrally for a capillary tube of outer bore 10 mm., lumen 0.90 to 0.95 mm., and overall length 20 cm. The joint between capillary and glass plate is a tapered ground joint; the end of the capillary and the surface of the glass plate are ground together so that both lie in the same plane.

The middle and top pieces of the osmometer are made of lucite. No washers are used in the osmometer proper.

The material placed under the membrane and directly on the glass plate is a disc of silk bolting cloth No. 20 or No. 25 Standard.[4] If the disc is not slightly smaller than the opening in the middle piece of the osmometer, tight sealing of the membrane will be impossible. Used discs last for months in water, saline, or Ringer's containing thymol. The new cloth as received may be greasy and is satisfactorily cleaned with Dreft in lukewarm water.

The metal collar is hollowed out to serve as a water jacket so that all observations may be made at one temperature. Correction by computation of measurements made at various temperatures to any one reference temperature is entirely unreliable for such fluids as serum (Hepp (9)). Temperature control to ±0.5°C. is satisfactory. The capillary should also be provided with a water jacket.

[3] Refers to apparatus made at the Harvard Medical School and covers only details lacking in Hepp's (2) paper.

[4] Obtainable from Tobler, Ernst & Traber, Inc., 71 Murray Street, New York City.

Smooth changes in the suction applied to the capillary are achieved by inserting one or two 20 cm. lengths of 0.2 to 0.3 mm. glass capillary into the rubber tubing connecting levelling bulb with manometer and manometer with osmometer capillary.

The levelling bulb is moved on a sprocket and chain arrangement by a geared wheel at table level. The bulb may conveniently be raised or lowered less than a millimeter at a time.

Operation[3]

The fixed part of the metal collar is turned upside down on a swivel support by rotation through 180°.

A disc of bolting cloth wet with the appropriate solution is placed on the ground surface of the glass plate inside a pencilled guide circle. The membrane, previously soaked in the appropriate solution, is placed on top of the cloth. Its top surface is carefully dried with a sheet of hard filter paper.

The middle piece of the osmometer is placed on the glass plate. Both parts with the included membrane are inverted and set into the metal collar. A circular rubber washer about 2 mm. thick is placed on top of the glass plate, the thin separate plate of the metal collar on top of the washer; then the two parts of the collar are approximated by uniform tightening of four bolts.

The osmometer is rotated through 180° to its final position. The solution to be filtered through the membrane into the capillary is placed on top of the membrane. A layer 1 mm. thick is sufficient. One may suck Ringer's solution, a true ultrafiltrate of the protein solution to be studied, or first Ringer's, then 1 or 2 cm. of true ultrafiltrate into the capillary.

The capillary is now inserted into the hole in the glass plate. No lubricant is used on this joint. A steel spring on the separate plate of the metal collar holds the capillary firmly in place.

The top piece of the osmometer is set in place. It serves as a cover which permits contact with air yet reduces evaporation.

The right-hand end of the capillary is connected to the manometer. Sufficient suction is applied to half fill the capillary with Ringer's, phosphate buffer, or ultrafiltrate at a slow steady rate so that 15 to 20 minutes will be required.

The solution in the osmometer is removed and the membrane carefully dried with pieces of filter paper until it appears dry to inspection and the column in the capillary begins to move toward the osmometer as a result of evaporation through the membrane.

The protein or colloid solution should then be placed in the osmometer. It may be covered with oil although in the time required for the usual measurement the evaporation taking place does not influence the result.

A convenient mark on the scale of the reading microscope is quickly selected and the previously applied suction reduced or increased to keep the meniscus of the capillary column at the mark. In 15 to 30 minutes the meniscus becomes steady and measurement may be begun.

When the free area of membrane used is 45 mm. in diameter it will be found that if the applied suction be increased, then reduced more than 1 mm. from its apparent equilibrium value, there will be produced a considerable movement of the meniscus of the capillary column, first in one direction then in the other. The osmotic pressure may be thus delimited within the range ± 1 mm. about the apparent equilibrium value.

Determination of the pressure by such a direct reading method may then be repeated in 30 minutes or less and will give a result within 5 per cent of the true equilibrium value as determined by interpolation.

When the free area of membrane used is 30 mm. in diameter, the direct reading method of locating the osmotic pressure can be used but it is considerably more tedious than with the larger membrane area unless the magnification used in the reading microscope is increased to 60×. The instrument can stand this increase in magnification. It is preferable, given the 30 mm. area and 40× magnification, to determine the equilibrium pressure by interpolation to 0 filtration rate from measurements of filtration rate at applied suctions ± 15 to 25 mm. about the apparent equilibrium value.

The desired suction is applied and after 30 seconds to 2 minutes the position of the meniscus is read on the microscope scale; then the position is read at 1 minute intervals for 2 to 6 minutes. Now the desired suction in the opposite direction from the apparent equilibrium value is applied and the readings taken as above. A little practice enables one to obtain fairly uniform filtration rates; only such rates are used in the plot. A plot is made of filtration rate in divisions per minute against applied suction and the suction corresponding to 0 filtration rate read off the resulting line.

Corrections

These are the depth of the layer of solution on the membrane (1 mm. or more), the distance from the membrane to the lumen of the horizontal limb of the capillary (20–21 mm.), and the capillarity correction for the fluid actually in the capillary.

SUMMARY

Estimation of the molecular weight of horse serum albumin from the osmotic pressure of solutions containing 0.713 to 5.12 gm. per 100 cc. shows that the Hepp osmometer yields the same values as the standard simple osmometer of Adair.

Accuracy and precision of the instrument decrease noticeably at concentrations of albumin less than 0.7 gm. per 100 cc.

A determination in duplicate can be carried out with this instrument in less than 2 hours.

The instrument is easily operated.

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STUDIES CONCERNING THE NATURE OF THE SECRETORY ACTIVITY OF THE ISOLATED RINGER-PERFUSED FROG LIVER

I. The Differential Secretion of Pairs of Dyestuffs*

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INTRODUCTION

In previous experiments (Höber and Titajew (1)) it has been shown that the secretory power of the isolated Ringer-perfused frog liver with respect to dyestuffs is exceedingly great. It surpasses by far that of the salivary gland of mammals and it is also markedly greater than that of the frog kidney, estimated from concentrations in the urine. Often, the concentration of dye appearing in the gallbladder cannula reaches a level several hundred times, sometimes even more than a thousand times, higher than in the perfusing solution. This has been observed with specimens of monoazo-, triphenylmethane-, azine- and oxazine-dyestuffs. In experiments on the isolated Ringer-perfused frog kidney one rarely observes a concentration stronger than 60 times. However, it has to be kept in mind that here the higher concentration of dyestuff is the result of the compound activity of the different parts of the nephrons. On one hand, the secretory power of the proximal tubules raises the concentration and on the other, the primary product of this tubular activity becomes more or less diluted according to the amount of added glomerular filtration. Therefore, it might appear questionable, whether, *de facto*, the concentrating power of the secretory elements of the kidney is inferior to that of the liver, and, indeed the microscopical picture of the tubules of the aglomerular kidney of the toadfish, that has been injected with certain dyestuffs, reveals a surprisingly intense coloration of the lumina, which seems to be indicative of a very high concentration (Höber (2)). In other respects the superiority of the secretory power of the isolated liver is beyond question. From previous and recent studies we know about 80 dyestuffs, which are

* It is a great pleasure to acknowledge the support to this work from the Penrose Fund of the American Philosophical Society.

highly concentrated by the liver, 33 of which fail to pass the tubular walls of the Ringer-perfused kidney. Besides those 80 dyestuffs secreted by the liver we have encountered 16 other dyes, which cannot pass this gland. Of these 16 dyes, 11 are highly colloidal, 2 of them are particularly highly lipoid-soluble and, evidently, owing to this property, they are retained in the liver fat.

Now, on the basis of these preliminary observations, we have tried to investigate the unknown mechanism of secretory activity of the liver, (1) by supplying the liver with mixtures of pairs of dyestuffs, in order to learn whether and for what reason one dyestuff may undergo preferential transport before the other; (2) by determining whether by the presence of additional substances the active secretion could be either enhanced or diminished in a reversible manner, and (3) by studying, whether, apart from the dyestuffs, other substances are equally suitable for secretory accumulation in the bile.

Method

This and the following paper include earlier and more recent work. In the earlier work *Rana temporaria* was used, in the recent *R. pipiens*. Differences in the behavior of these two species have not been remarked. The operative procedure has lately been described by Haywood and Höber (3). 0.0005 per cent of dyestuff was added to the perfusion fluid. When a mixture of two dyes was perfused, each concentration was determined with an approximate accuracy by employing a Walpole comparator. The mixtures were composed of red (or orange) and blue dyestuffs. In about 200 experiments 16 red and 5 blue dyestuffs have been compared with each other.

EXPERIMENTS

When the liver was perfused with a mixture of two dyestuffs, in general, the dyestuffs reappeared in the secretion in a ratio more or less different from that in the perfusion fluid. In the majority of our experiments, both dyes became strongly concentrated by the surviving gland. In Table I a few of our experiments are summarized. Usually the experiments were divided into three periods (I, II, III), each of them running over 1 to 3 hours. The numbers in the table indicate the dyestuff concentrations in relation to the original concentration (concentration ratios).

In the first six of the reported experiments the concentration of one dyestuff prevailed throughout the experiment over that of the other. In a smaller group of experiments, two examples of which are shown (VII, VIII), the initial concentration difference disappeared later. In a third smaller group (see IX and X) the initial concentration ratio was reversed.

Not only in experiments like those mentioned, but also when the liver was supplied with one dyestuff only, the concentration effected by the liver

varied a great deal from one experiment to the other (Höber and Titajew (1)). However, in the first periods of our experiments with mixtures, the relationship between the concentrations of the dyestuffs showed a high degree of regularity; *e.g.*, from a mixture of orange R and eriocyanin A with-

TABLE I

Experiments Showing the Differential Secretion of Pairs of Dyestuffs

	Dyestuff	Concentration ratio				Dyestuff	Concentration ratio		
		I	II	III			I	II	III
I	Ponceau 2R	500	175	—	VII	Erythrine P	120	165	—
	Cyanol	15	21	—		Cyanol	15	165	—
II	Patent blue V	1660	1260	440	VIII	Erioglaucin A	220	600	165
	Phenol red	25	19	7		Azofuchsin I	110	600	165
III	Palatine scarlet A	200	660	60	IX	Eriocyanin A	500	1000	82
	Erioglaucin A	48	165	30		Azofuchsin I	125	1000	165
IV	Erioglaucin A	100	330	—	X	Indigo carmine	165	140	40
	Erythrine P	12	40	—		Erythrine P	40	140	150
V	Patent blue V	332	800	440					
	Acid fuchsin	40	24	26					
VI	Rosinduline 2G	3200	2000	2400					
	Eriocyanin A	192	120	300					

TABLE II

Relative Speed of Dyestuff Passage through the Liver

I	Tropeoline 000/2, orange R, ponceau 2R, palatine scarlet A, orange GT, rosinduline 2G
II	Patent blue V
III	Rosinduline 2B
IV	Erioglaucin A
V	Brilliant orange RN
VI	Eriocyanin A
VII	Azofuchsin GN
VIII	Indigo carmine
IX	Phenol red, orange G, brilliant crocein 3B, azofuchsin I, erythrine P, azofuchsin IV
X	Cyanol
XI	Acid fuchsin, azofuchsin S.

out exception orange R was concentrated more than azofuchsin I, rosinduline 2G more than indigo carmine, and so on. The results of these comparisons are summarized in Table II.

Still another kind of effect has been observed, which seems to be of particular interest. The accumulation of a certain dye may be suppressed

largely or completely by the addition to the perfusion fluid of a second dyestuff, which itself may be strongly concentrated. For instance, Table III shows the results (1) of four experiments (*a–d*), which have been performed simultaneously with the livers of four frogs of the same size, and (2) of two corresponding experiments (*a* and *b*) on two livers.

Obviously, the secretion of indigo carmine is suppressed by orange GT and orange R, but not by azofuchsin I, and the secretion of phenol red is suppressed by eriocyanin A. In our experiences, those dyestuffs in particular, which belong to group I in Table II, are apt to prevent the concentration of a second dyestuff.

TABLE III

The Blocking of the Secretion of One Dyestuff by Another

Dyestuff	Concentration ratio		
	I	II	III
a. Indigo carmine	180	240	500
b. Orange GT	500	500	395
Indigo carmine	20	0	0
c. Orange R	120	500	1000
Indigo carmine	7.5	30	0
d. Azofuchsin I	80	125	167
Indigo carmine	167	250	330
a. Phenol red	80	90	30
b. Eriocyanin A	80	100	24
Phenol red	10	8	0

DISCUSSION

Separation of two dyestuffs by glandular activity has been observed previously. Liang (4) has shown that two dyestuffs, *e.g.* phenol red and cyanol, perfused together through the frog kidney from the renal portal vein, are quantitatively separated, only phenol red appearing in the urine, unless the pressure in the portal vein is raised so much that the perfusion fluid enters the glomeruli. The simple interpretation is that cyanol as well as phenol red passes the glomerular filter, whereas phenol red alone traverses the tubular wall and becomes concentrated (Höber (5)). When the intact frog is injected with phenol red-cyanol, at first phenol red prevails in the urine, but later—comparable to the situation in experiments IX and X (Table I)—cyanol predominates (Steffanutti (6)). The chief reason is that, in the beginning, concentrated phenol red and filtered cyanol enter the urine, while, after the major part of phenol red has been eliminated from the body, the cyanol becomes predominant. However, these results

with the kidney are not analogous to those described for the liver, since, probably, in the liver the separation is not brought about by the activity of different structural units, like glomeruli and tubules, but is somehow attributable to special unknown differentiating capacities of the single liver cell. Possibly similar capacities could be proved to exist in the tubular epithelia by providing them from the portal vein with a mixture of dyestuffs, each of which could be secreted. However, experiments of this kind have not yet been performed.

Now, it is important to know, why one dyestuff appears earlier or more abundantly in the liver secretion than another. Certainly, this question touches on the fundamentals of selective absorption and secretion, which doubtless are complex phenomena. The experiments described above are suggestive in considering, whether the first step of absorption and secretion, namely the entrance of the substances in question, may be correlated with some simple physicochemical properties such as diffusibility, lipoid solubility, adsorbability.

The diffusibility of our dyestuffs was studied by placing in test tubes 5 cc. of 0.1 per cent solutions of dyestuffs above 10 cc. of a gel of either 5 per cent or 10 per cent gelatin or of 3 per cent agar and by observing for several days the migration of the dyes into the gels. The result was that the concentration ratios of one dyestuff in a mixture to the other in the first period of secretion (Table II) are by no means related to the order of the diffusion rates, *e.g.* in gelatin: patent blue V > cyanol > acid fuchsin, eriocyanin A > indigo carmine > erythrine P > ponceau 2R > azofuchsin S and in agar: patent blue V > cyanol, acid fuchsin > eriocyanin A > erythrine P, ponceau 2R > indigo carmine > azofuchsin S.

Also lipoid solubility is found not to be a factor in the differential secretion of the first period, as *e.g.* orange R and tropeoline 000/2 are lipoid-soluble, ponceau 2R and palatine scarlet A are not.

In contrast to lipoid solubility and diffusibility there are some indications that adsorbability might have some bearing upon the initial steps of the process of secretion. It has been mentioned (p. 185) that, in comparison to the ability of the liver, only a restricted number of dyes can be taken up and transported by the tubules of the Ringer-perfused frog kidney. Now, in experiments, which will be published elsewhere, it has been found that in several groups of acid azo-dyestuffs only those are allowed to pass the tubular wall, which are endowed with a polar-non-polar configuration of their molecules. This could be accounted for by assuming that at the interface between cell and surroundings the dye first has to be anchored to one medium by the hydrophilic polar half of its molecule, to the other by the organophilic non-polar half before it can enter the cell. Lack of such

a polar-non-polar structure as well as a surplus of hydroaffinity conferred by the presence of some additional strong polar groups prevents the formation of such an interfacial layer of dye. We have not yet performed enough experiments on the liver with substances of identical basal structure to suggest that interfacial forces might also initiate the dyestuff secretion in this gland. As yet, the only hint of such an effect is that, when a great number of sulfonic acid groups (4 to 5) is present in the molecule presented to the liver cell, no secretion occurs. The conclusion is that the overwhelming influence of these strongly hydrophilic groups prevents the initiating attachment to the cell surface. Dyestuffs of this kind are ponceau 6R, azofuchsin V and an azo-dyestuff of the I. G. Farbenindustrie A.G. with five sulfonate groups.

Finally, it should be noted that our observations regarding the prevention of the secretion of one dyestuff by the presence of another one are suggestive of the idea that in the liver the same mechanism controls the transport of two diverse substances. This would be different from the active transport in other systems. For instance, the absorption of amino acids, which takes place simultaneously with that of glucose, remains unaffected, after the poisoning of the intestine with phlorizin has inhibited the glucose absorption. The same is true of the reabsorption of chloride and of glucose by the frog kidney.

SUMMARY

The isolated Ringer-perfused frog liver is able to concentrate in its secretion several hundred or even thousand times a great number of dyestuffs. When two dyestuffs are perfused simultaneously, the liver separates them to a lower or higher degree, some times to such an extent that the secretion of one of them is entirely suppressed by the other. Which one of the two dyestuffs appears prevailingly in the secretion, does not depend upon its diffusibility or lipoid solubility, but perhaps upon its adsorbability. This is concluded from the fact that dyestuffs provided with several (4 to 5) strongly hydrophilic sulfonate groups in their molecule, are not permitted to pass the gland.

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STUDIES CONCERNING THE NATURE OF THE SECRETORY ACTIVITY OF THE ISOLATED RINGER-PERFUSED FROG LIVER

II. The Inhibitory and the Promoting Influence of Organic Electrolytes and Non-Electrolytes upon the Secretion of Dyestuffs*

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INTRODUCTION

As described in the preceding paper (Höber (1)), the isolated Ringer-perfused frog liver is characterized by its ability to collect from the circulating fluid a great number of dyestuffs to such a degree that they appear in the secretion many hundred times concentrated. Now, this activity of the surviving gland can be abolished temporarily in a very striking way by substituting in the perfusion fluid 1/8 to 1/10 of the NaCl with the isosmotic amount of certain organic non-electrolytes or electrolytes. After a short time the color of the secretion fades, often completely, but reappears after resumption of perfusion with Ringer (Höber (2), Valdecasas (3)). Non-electrolytes producing this inhibitory influence are chiefly the disaccharides, hexoses, pentoses, and hexahydric, pentahydric, and lower polyhydric alcohols as well as amino acids and succinamide. Electrolytes acting in the same way are the sodium salts of the lower fatty acids (acetate, propionate), of lactic acid, and of amino dicarboxylic acids. It appears that all these inhibitory substances have a strong hydropolarity and therefore lack surface activity. The opposite action, promotion of the secretory power of the liver, has been known for a long time to be exerted by salts of the bile acids. A similar effect has been observed with some anesthetics, with saponin, and with salts of some organic bases and of some higher fatty acids. In contrast to the group of inhibiting agents these substances are characterized by surface activity. Investigation of their influence pre-

* It is a great pleasure to acknowledge the support to this work from the Penrose Fund of the American Philosophical Society.

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sented some difficulty, since they cause irreversible loss of activity, unless the concentrations are kept below a low, somewhat variable limit and unless the application is restricted to a short and likewise variable period. Further investigation of these phenomena seemed to be desirable in order to throw more light upon the unknown mechanism of one of the main features of glandular activity.

EXPERIMENTS

I. Inhibition of the Secretion of Different Dyestuffs

Since the earlier experiments were concerned with only a small number of dyestuffs, we decided to extend the work in order to determine whether any generalization could be made concerning the relationship of the physicochemical character of the dye to the aforementioned inhibitory effect. Table I summarizes four experiments with (1) a basic dye, (2) a lipoid-insoluble acid dye, (3) a lipoid-soluble acid dye, and (4) a mixture of one lipoid-soluble and one lipoid-insoluble acid dye. Each dye was injected into the liver through the abdominal vein at a concentration of 0.0005 per cent, as described in the previous paper (1).[1]

TABLE I

The Inhibitory Effect of Mannitol and of Sucrose

	I	II	III	IV	V	VI
1. Rhodamin 3B (+ 0.027 mol mannitol)	250	250	**125**	**60**	**80**	250
2. Eriocyanin A (+ 0.027 mol mannitol)	250	**180**	**60**	**0**	375	
3. Orange R (+ 0.027 mol sucrose)	4000	4000	**2000**	**90**	**60**	500
4. Orange R (+ 0.027 mol mannitol)	120	**0**	167			
Patent blue V	120	**0**	81			

From these and a great number of analogous experiments it appears that neither lipoid solubility nor the acidic or the basic character of the dyestuff has a special bearing upon the result.

The inhibitory action of the aforementioned organic substances might be thought of as attributable to alterations in the circulatory conditions. As a matter of fact, the decrease in dyestuff concentration is usually associated with a fall in the amount of secretion. However, this decrease is by no means invariably connected with a diminished perfusion rate, but,

[1] The figures in Table I indicate the concentration ratios during the experimental periods I to VI, each of which extended over one-half to one hour. The numbers in bold faced type are the concentration ratios observed in the presence of the non-electrolytes indicated.

on the contrary, appears to be associated with a more rapid as well as a slower perfusion (Table II). A definite connection between circulation and dyestuff concentration in the secretion does exist as can be shown by simply changing the perfusion pressure without the addition of any of the organic substances. Raising the pressure raises the amount of secretion and diminishes the concentration ratios; reducing the pressure has the opposite effect (Table II). By these and many other data we are led to conclude that the inhibitory action of the organic substances applied must be looked upon as a direct influence upon the secretory structure and not as being brought about indirectly by alteration of the circulatory conditions.

TABLE II

Perfusion Rate and Secretory Activity

		Perfusion	Secretion	Concentration ratio	Dye secreted mg./hr. × 10^4
		cc./hr.	*mg./hr.*		
Eriocyanin A	—	200	3	198	30
"	+ 0.027 mol adonitol	80	1	60	3
"	—	120	4	500	100
Ponceau 2R	—	65	10	240	120
"	+ 0.027 mol mannitol	75	8	62	25
"	—	52	12	660	400
Orange R	—	60	5.7	5350	1525
"	+ 0.009 mol succinate	60	2	13	1.3
"	—	70	5	1080	270
Eriocyanin A	at 10 cm. H_2O pressure	48	2.5	800	100
"	20 "	198	9	40	18
"	10 "	92	2.5	200	25

Attention had been turned by previous work (Höber and Titajew (4), Valdecasas (3)) to the fact that those organic electrolytes and non-electrolytes which appear to inhibit the liver activity are characterized by common chemical and physicochemical features, which may be essential for their action; namely, the possession of polar groups, —COOH, —OH, and —NH_2. For this reason, the molecules are strongly hydropolar, surface-inactive, and lipoid-insoluble and they exhibit anti-dispersing, consolidating properties toward hydrophilic colloids. In recent experiments we have extended our study to the Na salts of some markedly hydropolar acids, to malonate, succinate, glutarate, gluconate. They appeared

to exhibit vigorous but reversible effects (for the effect of succinate see Table II), whereas glycolate displayed a considerably weaker inhibiting influence.

II. Organic Substances Promoting Liver Secretion

The salts of the bile acids are the only substances which it is generally agreed show a definite choleretic power. Their essential function in the intestine seems to rest upon their hydrotropic properties (Neuberg (5)), *i.e.* the capacity to bring into aqueous solution substances which otherwise

TABLE III

Effect of Benzene Sulfonate at Various Concentrations

Mol benzene sulfonate	Rate of secretion	Concentration ratio	Dye secreted mg./hr. × 10^4
	mg./hr.		
—	1.25	400	25
0.22×10^{-2}	2	250	25
"	4	500	100
"	4	750	150
—	5	600	150
—	1	500	25
0.45×10^{-2}	2	500	50
"	7	300	110
"	4	500	100
—	6	500	150
—	1	500	25
0.9×10^{-2}	2	125	13
"	2	120	12
"	3	40	6
—	4	250	50
—	2	600	60
3.6×10^{-2}	3	300	45
"	5	80	20
—	6	30	9

are scarcely soluble, like higher fatty acids and sterols. This is brought about by their polar-non-polar structure, the organophilic portion of the molecule being strongly attached to the undissolved substance, the hydrophilic portion pulling the substance towards the water. This concept was an incentive to testing other hydrotropic substances for possible choleretic power. In this respect, we have studied the Na salts of fatty acids with carbon chains longer than C_8, of benzoic and oxybenzoic and of aromatic sulfonic acids. Because of their surface activity and lipoid-solvent proper-

ties, we also have investigated the effects of some anesthetics and alkaloids, of saponin and digitonin, and other related substances. All of these, above certain concentrations and exposure periods, produce or at least promote cytolysis, by favoring disintegration of the surface structure of cells. For this reason, it was very difficult to secure unequivocal and uniform results and to avoid irreversible damage.

In a series of several hundred experiments, substances of this group were applied in varying concentrations and for varying periods of time until

TABLE IV

Effect of Caprylate at Various Concentrations

Mol caprylate	Rate of secretion	Concentration ratio	Dye secreted mg./hr. $\times 10^3$
	mg./hr.		
—	15.2	930	71
3×10^{-5}	14.1	1120	78
—	10.2	1310	67
—	11.0	390	21
—	5.5	3200	89
3×10^{-5}	6.6	1090	36
—	8.0	7000	280
—	7.2	2270	81
—	8.6	320	14
6×10^{-5}	11.6	260	15
—	8.4	330	13
—	11.4	2520	140
3×10^{-4}	5.0	900	22
—	2.4	120	14
—	3.85	140	2.7
6×10^{-4}	3.25	100	1.6
—	3.15	50	0.8

conditions were found in which the stimulating action was preponderant over the toxic. For some substances such conditions were never found. Frequently even with supposedly optimum concentration and duration of experiment toxicity was evident, especially during warm weather and following starvation attendant upon hibernation. For this reason and bearing in mind the fact that the isolated Ringer-perfused liver is, in any case, a dying organ, we felt justified in discarding all experiments in which the liver was evidently moribund as indicated by the progressive loss of secretory power.

The variability in the course of our "good" experiments is illustrated by Tables III, IV, and V.

In Table III the results of four experiments are reported, in which sodium benzenesulfonate in various concentrations was added to the Ringer solution. 0.9×10^{-2} mol and 3.6×10^{-2} mol cause a definite diminution of the secretory power, while 0.45×10^{-2} mol and 0.22×10^{-2} mol produce a promoting effect. This is particularly demonstrated by the data regarding the absolute amounts of dyestuff secreted per hour.

The series of six experiments described by Table IV displays the more complicated behavior of the liver when subjected to the influence of Na caprylate. The poisoning effect of the higher concentrations (3×10^{-4} and 6×10^{-4} mol) is evident. However, with the markedly lower concentration of 3×10^{-5} mol a promoting effect is produced, which is characterized by its appearance only after a certain lapse of time.

TABLE V

Substances Expected to Have a Promoting Influence upon Liver Secretion

	Optimal concentration	Concentration ratio	Dye secretion/hr.
Caprylate	3.10^{-5} to 6.10^{-5}	4+, 2−	4+, 1−
Heptylate	1.10^{-5}	1+, 2−	3+
Oleate	5.10^{-6}	4+, 2−	4+, 2−
Benzoate	$0.5.10^{-2}$	3+	2+
Salicylate	7.10^{-5}	5+, 2−	6+, 1−
Benzenesulfonate	$2.2.10^{-3}$ to $4.5.10^{-3}$	2+	2+
p-Toluenesulfonate	6.10^{-4}	3+, 1−	3+, 1−
Glycocholate	3.10^{-5} to 20.10^{-5}	3+, 2−	5+
Taurocholate	3.10^{-5}	5+, 1−	6+
Propylcarbamate	$0.6.10^{-2}$ to 5.10^{-2}	4+, 1−	4+, 1−
Codein hydrochloride	3.10^{-4} to 15.10^{-4}	4+	4+
Veratrin	5.10^{-6}	1+, 1−	2+
Digitonin	$0.5.10^{-4}$ to 1.10^{-4}	3+, 1−	2+, 2−

Table V is a survey of a series of experiments with substances expected to show a promoting influence on the basis of their polar-non-polar structure, their surface activity, and their lipoid solubility. In more than 70 per cent of the experiments the substances tested in concentrations which seemed suitable were found to stimulate the secretory capacity.[2]

On the other hand, with substances likewise exhibiting the aforemen-

[2] In Table V a plus sign means presence, a minus sign, absence of a promoting action reflected by the concentration ratios or the absolute amount of dyestuff secretion or by both of them.

tioned physicochemical properties, we have failed as yet to secure a promoting action. Those substances were nonylate, laurate, cinnamate, and β-naphthalenesulfonate.

III. Antagonistic Effect of Two Substances of Opposite Action upon the Dyestuff Secretion

The failure of the experiments dealt with in the preceding section, to yield clear cut results was believed to be due to the fact that there is a gradual transition in the influence of the organic substances from a mere loosening to a definitely disintegrating effect. This interpretation seems to be supported by observations dealing with the neutralization of the vigorous inhibitory influence of one organic substance by the addition of a

TABLE VI

Antagonistic Effect of Inhibiting and Promoting Substances

	Concentration ratio	Secreted dye mg./hr. × 10^4
Ringer	99	10
" +⅛ isotonic sucrose	0	0
" " +8.10^{-6} mol taurocholate	364	130
Ringer	500	100
" +⅛ isotonic sucrose	0	0
" " +2.5.10^{-2} mol diethylurea	165	8
Ringer	148	19
" +⅛ isotonic sucrose	>0	>0
" " +8.10^{-6} mol oleinate	250	25

second one. This behavior is demonstrated by experiments given in Tables VI and VII.

Table VI surveys three experiments in which substitution of 1/8 of the NaCl in the Ringer solution with 1/8 isotonic sucrose was succeeded by the entire or practically entire disappearance of the dyestuff from the secretion. Following addition of one of the polar-non-polar substances in a concentration found previously to be promoting the dyestuff secretion was resumed. The same antagonistic influence has been observed with the following compounds: Na glycocholate 9 × 10^{-6} mol, phenylurea 1 × 10^{-3} mol, propyl alcohol 1 × 10^{-2} mol, and caprylic acid 7 × 10^{-4} mol. When substances showing neither polar structure nor a distinct surface activity nor lipoid solubility were tested, it was found that glucose, on one hand, is unable to restore the secretory activity which has been abolished by lactose,

but that urea, acetamide, and ethylene glycol can definitely do so (Table VII). In general, these and other experiments have demonstrated that the inhibitory influence of disaccharides and hexoses is not diminished by other disaccharides, hexoses, and hexahydric alcohols, but is by urea, thiourea, methylurea, acetamide, propionamide, ethylene glycol and to some degree and more or less irregularly by glycerol and arabinose.

TABLE VII

Antagonistic Effect of Substances with Higher and Lower Hydropolarity

	Concentration ratio	Secreted dye mg./hr. × 10^4
Ringer	165	25
" +⅛ isotonic glucose	11	4.5
" " +⅛ isotonic urea	250	50
Ringer	400	100
" +⅛ isotonic sucrose	15	0.35
" " +⅛ isotonic acetamide	1000	42
Ringer	500	25
" +⅛ isotonic lactose	15	0.75
" " +⅛ isotonic glucose	3.7	0.25
Ringer	375	75
" +⅛ isotonic maltose	12	0.75
" " +⅛ isotonic ethylene glycol	185	9

IV. Physicochemical Properties of the Inhibiting and the Promoting Compounds

It has already been mentioned that those organic substances which show a marked inhibitory effect, exhibit a great affinity to water, which prevents their entering a lipoid phase, opposes their anchorage on the interface between water and organic matter, causes them to compete with the water molecules attached on hydrophilic colloids, and confers upon them in this way a shrinking influence. The promoting substances, in general, display opposite properties. Inasmuch as they show a polar-non-polar molecular configuration, the hydrophobic portion of their molecule can adhere to organic substances, the hydrophilic portion pulling towards the water and exerting in this way a loosening and swelling effect. However, there are also present in this group of promoting substances more or less apolar compounds which, because of their organophilic character as a whole, penetrate a lipoidal surface structure, change the intermolecular adhesive forces, and counteract the close molecular packing so that the final result is disintegration. Between these two contrasting groups are intermediate sub-

stances which either show only a slight hydroaffinity, like ethylene glycol and glycerol, or a slight organotropy, like acetamide and urea.

This interpretation of the influence of the two chief groups of organic compounds is supported by the following experimental facts: Katz (6) has investigated the effect of numerous organic substances upon the swelling and shrinking of starch. Of particular interest in connection with the present work was his observation of a shrinking effect with disaccharides and monosaccharides and with polyhydric alcohols in the order: mannitol, erythritol, glycerol, ethylene glycol. In contrast, some swelling is brought about by acetamide and urea and a stronger swelling by propyl and butyl carbamate. In his work with electrolytes, he observed marked shrinking with oxalate, malonate, succinate, glutarate, and citrate. A smaller effect was obtained with lactate and glycolate and with salts of the lower fatty acids (C_2 to C_4), whereas those of the higher members of this series (C_5 to C_9) produce swelling. This is true also with benzoate and still more with salicylate and with the salts of aromatic sulfonic acids.

However, model experiments with starch can hardly be comparable to the colloidal processes taking place on the surface of cells. For this reason, we have investigated the influence of the organic substances on lecithin, which seemed to provide a more appropriate model for cellular processes. A 0.2 per cent suspension of egg yolk lecithin in water or in 1 per cent NaCl was prepared. The pH was usually controlled with 0.04 mol phosphate buffer.[3] The increase (+) or decrease (−) in transparency following the addition of organic substances was measured by a photocell. An increase indicates a dispersing, a decrease an aggregating effect (Table VIII).

It is obvious that the results summarized in Table VIII are again confirmatory of the assumption that the dispersing and the antidispersing properties of the organic non-electrolytes and electrolytes are more or less correlated with their physiological effects.

Quite in line with our results are the observations of von Kuthy (7). Gelatin, like starch and lecithin, undergoes swelling in presence of hydrotropic substances like Na benzoate, benzenesulfonate, phenylacetate. The same substances facilitate the penetration of gelatin by dyestuffs.

Finally, with regard to the experiments on dyestuff secretion by the liver, Valdecasas (3) made the pertinent observation that solutions of a number of organic substances in the same concentrations in which they are found to increase the secretory activity, increased the rate of filtration through the hog's bladder.

[3] In order to avoid the influence of hydrolysis, the effect of the salts of weaker acids was, in general, studied at a higher pH (about 7.5 to 8) than that employed with the other substances.

In concluding, it should be emphasized that, although it can hardly be questioned that colloidal processes are involved in the physiological events, the importance of this statement must not be overestimated. Even such

TABLE VIII

Influence of Organic Substances on the Transparency of Lecithin Suspensions

	Effect	Transparency	pH
Sucrose	−	Minimum at 0.1 m.	4 to 4.6
Lactose	−	" 0.25 m.	4 to 4.4
Glucose	−	" 0.5 m.	4.2
Mannitol	−	" 0.1 to 0.4 m.	5
Erythritol	−	Flat minimum at 0.5 m.	4.2
Glycerol	−	Weak effect at 0.2 to 1.0 m.	4.2 to 4.4
Ethylene glycol	+	Increase of transparency above 0.1 m.	4 to 4.2
Glycine	−	Decrease " 0.1 to 1.0 m.	5.6 to 5.8
Amino butyric acid	−	" " 0.05 to 1.0 m.	7.2
Oxalate	−	" " 0.01 to 0.2 m.	4.2 to 4.4
Succinate	−	Strong decrease of transparency 0.02 to 0.2 m.	4 to 4.4
Glutarate	−	Minimum at 0.3 m.	5
Glycolate	−	" 0.5 m.	4.2
Malate	−	Rather strong decrease 0.01 to 0.08 m.	4
Capronate	+	Little effect up to 0.4, above 0.4 m. small increase	8 to 8.2
Heptylate	+	Increase 0.05 to 1.0 m.	7 to 8
Caprylate	+	" 0.005 to 0.04 m.	7.4 to 7
Pelargonate	+	Strong increase 0.01 to 0.8 m.	8.5
Oleate	+	Increase 0.01 to 0.1 m.	8 to 8.4
Benzoate	+	Strong increase 0.1 to 1.0 m.	7.8 to 8
Salicylate	+	" " 0.1 to 1.0 m.	4.5 and 8
Glycocholate	+	" " up to 0.01 m.	6
Taurocholate	+	" " up to 0.01 m.	6
p-Toluenesulfonate	+	Increase 0.02 to 0.8 m.	5
β-Naphthalenesulfonate	+	" 0.02 to 0.15 m.	4.5
Propylcarbamate	+	Small increase 0.03 to 0.4 m.	4
i-Amylcarbamate	0	No effect 0.0002 to 0.0012 m.	4.4
n-Butyl alcohol	+	Increase 0.18 to 0.65 m.	4
i-Amyl alcohol	+	Very small effect 0.005 to 0.08 m.	4.2
n-Heptyl alcohol	>0	" " 0.0005 to 0.007 m.	4.6
Veratrin	+	Strong increase 0.01 to 0.08 m.	7

striking results as those regarding the antagonistic effect of pairs of organic compounds, which belong to one and to the other of our main groups, fail to give us an insight into the secretory mechanism beyond the conclusion that a certain consistency and stability of the active cellular structure is indispensable.

V. Some Remarks about the Mechanism of the Dyestuff Secretion

In the following discussion the rate of secretion and the concentration of dyestuff will be considered separately. It has been pointed out earlier that, in general, on the one hand, a reversible decrease of the concentrating capacity of the liver, brought about by the addition of one group of organic substances, is accompanied by a decrease in the rate of secretion (Table II) and that, on the other hand, a reversible increase of concentration produced by the other group is often (although not regularly) associated with an increase in the rate (Tables III and IV). An irreversible increase of the rate of secretion is ordinarily observed after an overdose of a promoting substance has been given (Table III and also Valdecasas (3)).

The observations concerning the behavior of the rates might be interpreted as indicating some consolidating or shrinking effect of one group, some dispersing or loosening effect of the other group of substances. This would result in a greater or lesser permeability of the liver cells to water, in other words, in a narrowing or widening of their porous structure. Such a concept would be supported by recently published potentiometric measurements of Höber, Andersch, Höber, and Nebel (8) on muscle and nerve membranes. The membrane potentials appeared to be acted upon by the two groups of organic substances in opposite directions, indicating either an increase or a decrease in porosity or, respectively, a decrease or an increase of selective ion permeability.

With respect to the concentration of the dyestuffs, it is suggestive for several reasons, to regard their entrance into the liver as a matter of adsorbability. Two points may be emphasized here. (1) Apart from dyestuffs no substance has been definitely shown to be concentrated in the liver secretion. It is true that the passage of solutes through the liver has not, as yet, been thoroughly investigated. However, except for the dyestuffs, the substances which, up to the present, have been shown to pass the liver, pass it as they would a passive filter, *i.e.* without a marked change in concentration, and are characterized by their lack of adsorbability (Haywood and Höber (9)). (2) Apart from colloidal dyestuffs, which have been mentioned earlier (Höber (1)) as being unable to penetrate the liver, and apart from lipoid-soluble dyestuffs, which could be retained in liver fat, only a few diffusible and lipoid-insoluble dyes fail to find a pathway through the liver. From a series of about 30 azo-dyestuffs only those were not secreted, which contain in their molecule 4 to 5 sulfonate groups, azofuchsin V, Ponceau 6R, and a benzene-azo-naphthalene-pentasulfonate of the I. G. Farbenindustrie A. G. This may be explained by taking into account the great hydroaffinity of the sulfonate groups, which resist the

adhesive forces between the cells and the organophilic portion of the dye molecule. On the basis of such a concept one might infer that under the influence of the organic compounds the efficiency of the factors concerned in establishing the high concentration ratios can be varied, in one or the other direction, either by improving or impairing the accessibility to the dyestuffs of some cellular carriers, where the adsorption takes place, or by increasing or diminishing the rate of the carrier transport (Brinkman and von Szent-Györgyi (10) and Rosenthal (11)).

SUMMARY

The ability of the isolated Ringer-perfused frog liver, to concentrate dyestuffs in its secretion several hundred times, can be abolished entirely and reversibly by replacing in the Ringer solution about 1/8 of the NaCl by the isosmotic amount of a surface-inactive non-electrolyte (disaccharide, hexose, pentose, polyhydric alcohol, amino acid, acid amide) or electrolyte (salts of lower fatty acids, hydroxyl carboxylic, and dicarboxylic acids). This effect is not dependent upon changes in the perfusion rate.

The opposite effect, promotion of secretory activity, can be brought about by polar-non-polar electrolytes (salts of higher fatty acids, bile acids, and other aromatic carboxylic acids, aromatic sulfonic acids) and surface-active non-electrolytes (anesthetics, alkaloids, digitonin). However, reversibility of this effect cannot be regularly observed, since cytolysis is frequently the end result.

Suitable concentrations of inhibitory and promoting substances, simultaneously applied, counteract each other.

Inhibitory and promoting substances, in general, exhibit opposite effects upon the dispersion of colloids (starch, lecithin, gelatin).

The correlation between the physicochemical and the physiological action of the organic compounds is discussed.

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STUDIES ON THE PURIFICATION OF BACTERIOPHAGE

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Many of the earlier studies on the purification of bacteriophage were undertaken with the object of demonstrating that phage is an autonomous, organized antigen distinct from the bacterium at whose expense it regenerates. Other investigators were inclined to consider the active agent as a chemical substance rather than a living organism and attempted to obtain the active agent in a pure state in order to study its chemical nature. The findings in these early studies failed to supply enough data to establish the chemical composition of bacteriophage, since no convincing criteria of purity were applied, nor could the activity of the purified preparation relative to the weight of the agent be ascertained (1–7). On the basis of the negative outcome of the usual protein color tests, most of these workers have come to the conclusion that phage is not protein in nature. However, the failure of a preparation to give protein color tests cannot alone be considered as a sufficient evidence to exclude the presence of a small amount of protein, especially when one is dealing with substances exhibiting activity in extremely low concentration.

A few workers did supply enough data to at least permit estimation of the activity corresponding to the nitrogen content of the preparation. Thus Kligler and Olitzki (8) purified a coliphage by adsorption on kaolin in acid solution with subsequent elution by rendering the reaction alkaline, and obtained a preparation with 0.014 mg. nitrogen and 10^9 active lytic units per cubic centimeter of concentrate (1.4×10^{-11} mg. of nitrogen per unit of lytic activity). Because the protein color tests were negative, they concluded that phage was not protein. Colwell (9), by extraction of "lysogenic" cultures with water, obtained a preparation containing 0.0027 mg. nitrogen and 10^7 units per cc. (2.7×10^{-10} mg. N per unit of lytic activity).

In 1927 one of us (10) attempted the purification of phage by fractional ultrafiltration and obtained as an end product a preparation which had the following composition:[1] C = 37.8 per cent; H = 5.74 per cent; N =

[1] Analyzed through the kindness of Dr. A. Elek of The Rockefeller Institute.

4.18 per cent; ash = 18.9 per cent. 360 liters of ultrafiltered phage of titre 10^6 active units per cc. yielded 7.7 mg. of dry residue, which corresponds to 1×10^{-12} mg. of nitrogen per active lytic unit. When concentrated to a syrupy mass, just before reducing to complete dryness, this concentrate retained practically all its original activity, and 1 cc. of this gave negative protein color tests, but positive copper reduction. When reduced to dryness the material was totally inactive, and the organic analysis given above was made on such inactive material. The analysis suggested that the material consisted either of a nitrosugar or of a polysaccharide contaminated with small amounts of nitrogenous material. However, there was still another possibility that could not be excluded, namely that this nitrogen might represent a small amount of protein contaminated with a relatively large amount of some non-nitrogenous material, and that the amount of protein present was insufficient to give protein color tests. This possibility seems the more likely in the light of recent observations of Northrop (11) that in his purified preparations of phage the active protein was contaminated with mucin-like carbohydrate which was difficult to remove completely.

Recent studies have indicated that phage might be protein in nature. Schlesinger (12–14) collected bacteriophage on a collodion membrane, purified it by repeated high speed centrifugation, and obtained a preparation which he described as having a density of 1.3 and consisting of spherical particles about 85 mμ in diameter. Calculated on the basis of the lytic activity of the original material, the dry residue represented 4.3×10^{-13} mg. of substance per unit of activity. Since the analysis of the residue showed that it contained 13 per cent of nitrogen, it appears that this preparation had 5.6×10^{-14} mg. of nitrogen per unit of lytic activity. Despite the fact that the Molisch test became very weak during purification, on the basis of the high phosphorus content (3.7 per cent), the positive Feulgen reaction, and insolubility in dilute alkali, Schlesinger concluded that it was a nucleoprotein. More recently Northrop (11), following the chemical procedure he has previously employed for purification of enzymes, isolated from a staphylococcus phage a nucleoprotein bearing the phage activity, and since hydrolysis of this nucleoprotein resulted in loss of activity, he considered that it represented the phage itself. He observed that this purified phage was quite labile and was quickly inactivated upon drying. About 5×10^{-13} mg. of this protein sufficed to produce lysis of a young culture of the homologous organism. On the assumption that this amount represented one molecule, he calculated the molecular weight to be about 300,000,000 and the diameter of the particles to be about 100 mμ. He

points out that "The active protein is of much higher molecular weight than most normal proteins and this is true of viruses in general."

On the other hand, by subjecting *B. coli* bacteriophage to fractional ultrafiltration, one of us (15) has shown earlier that particles endowed with lytic activity varied in size within wide limits but in general were of the order of magnitude of simple proteins (2 to 20 mμ in diameter). In attempting to explain this discrepancy, Northrop (11) investigated the diffusion rate of high diluted suspensions of his purified staphylococcus bacteriophage and found that it also contained smaller particles of about 5.6 mμ in radius corresponding to a calculated molecular weight of about 450,000. Since the small particles were apparently in equilibrium with the large ones (they were obtainable by simple dilution), he concluded that "... this protein exists in various sized molecules of from 500,000–300,000,000 molecular weight, the proportion of small molecules increasing as the concentration decreases."

In view of the evidence for the protein nature and relatively large molecular size of bacteriophage supplied by the experiments of Schlesinger (12–14), and particularly of Northrop (11), it was of interest to repeat the earlier (1927) experiments of Bronfenbrenner.

Methods

In our earlier experiments (1927) the Berkefeld filtrate of the lysed culture was forced through a relatively dense collodion membrane in order to remove the bulk of bacterial debris, and the ultrafiltrate was then concentrated on a double 7 per cent membrane. This procedure had two disadvantages: in the first place, the first ultrafiltration resulted in a considerable reduction in phage content so that the ultrafiltrate seldom contained more than 10^6 lytic units per cc. In the second place, collection of the ultrafiltered phage on a double 7 per cent membrane might have been responsible for the failure to remove some of the contaminating carbohydrate by subsequent washing. The more recent finding that bacterial protein is largely hydrolyzed during lysis (16–17), and the evidence that the particles of bacteriophage may reach the size of heavy protein molecules, suggested the possibility of collecting the phage directly on a collodion membrane of such a density that would just fail to let it pass without the preliminary fractionation.

Since the phage was to be collected directly on a collodion membrane, the medium of propagation obviously had to be completely diffusible through that membrane. To meet this requirement, the synthetic medium described by Hetler and Bronfenbrenner

(16) was used (modified to the extent that dextrose was substituted for saccharose). To avoid formation of a precipitate this medium was sterilized in two separate parts.

Part 1.—1 gm. Na_2HPO_4; 6 gm. ammonium succinate; 500 cc. distilled water. Titrate to pH 7.6. Autoclave.

Part 2.—20 gm. dextrose; 1 gm. $MgSO_4$; 1 gm. K_2SO_4; 500 cc. distilled water. Autoclave. When cool mix parts 1 and 2 and readjust to pH 7.2 with sterile NaOH.

The culture used throughout the work was a strain of colon bacillus (*B. coli* P.C.) which was known to be free of spontaneous lytic activity over a period of 15 years. This organism grew readily on the synthetic medium. When homologous phage (P.C.) was added to a young culture, regeneration of the phage was completed in 16–18 hours, though the culture was not sterilized so that in about 20–24 hours overgrowth of resistants usually became apparent.

All titrations of phage were carried out by a procedure which represents an adaptation of a method of counting bacteria described by McCrady (18), except when another procedure is indicated. This titration consists essentially in preparing serial tenfold dilutions of the active filtrate and testing five separate 1 cc. samples from each dilution for the presence of phage by addition of susceptible culture. As the dilution factor increases, the proportion of 1 cc. samples showing lysis will decrease. From data representing distribution of samples showing lysis (containing phage) the probable number of active units in the original suspension was calculated by means of probability tables (18).

The reinforced collodion membranes were prepared by depositing collodion[2] on the inner surface of an alundum thimble under pressure. In the preliminary experiments membranes of various densities were prepared by varying the concentration of the collodion and the time and pressure under which the collodion was deposited. The procedure which was chosen consisted of depositing the collodion under a vacuum of 15 inches of mercury for 1 minute, then emptying the thimble, and permitting it to drain for 2 minutes, after which it was solidified by admitting water and washed to remove the acetic acid for at least 24 hours before use.[3] These membranes were fairly reproducible as indicated by preliminary test of each membrane as to the rate of flow of water through it under pressure of 20 inches of mercury. All membranes which permitted the passage of a liter of water in about 1 hour and 15 minutes were found to be satisfactory.

EXPERIMENTAL

Approximately 16 hour *B. coli* P.C. phage lysate in synthetic medium was filtered through a Berkefeld filter in order to remove the bacteria and debris remaining after lysis. The phage was then concentrated by ultrafiltration on a 5 per cent collodion membrane, washed with distilled water until the filtrate was negative for ammonia

[2] The 5 per cent collodion solution was prepared according to the method of Bechhold and Gutlohn (19) and consisted of 50 gm. of nitrocellulose and 12.5 gm. of K_2CO_3 dissolved in 1000 cc. of glacial acetic acid. Incidentally, it should be noted that the membranes were prepared from a new batch of nitrocellulose, and were considerably less permeable than those prepared from a different lot of nitrocellulose in 1927.

[3] The essential details and precautions to be followed in this procedure have been described by Bronfenbrenner (20).

(Nessler's reagent), and finally concentrated on the membrane under vacuum to a few cubic centimeters. The concentrated phage was removed with a pipette and any phage adhering to the membrane was washed down by a stream of a few cubic centimeters of distilled water, the walls of the membrane were swabbed with a sterile cotton swab moistened with distilled water, and all phage thus collected was similarly removed with a pipette. This procedure was repeated two or three times with a fresh supply of distilled water and the phage so recovered was added to the original concentrate. As will be shown later, this procedure resulted in the recovery of practically all the phage as shown by the determination of the lytic activity, and of the nitrogen content (Tablès I and III).

Since the total nitrogen content (0.6 per cent ammonium succinate) of the medium was about 1 mg. per cc., and the phage content at the end of incubation approximately 5×10^9 lytic units per cc., the original lysate (before ultrafiltration) represented $(1 \div 5 \times 10^9)\ 2 \times 10^{-10}$ mg. of nitrogen per each active lytic unit. However, since this nitrogen represents primarily unutilized ammonia as well as synthesized bacterial protein, and only a fraction of it might represent phage itself, it is evident that purification consisting of copious washing out of more diffusible extraneous constituents must result in a diminution of the nitrogen content per unit of lytic activity in the final product. This was actually found to be the case when the nitrogen content of crude and purified phage and of the phage-free ultrafiltrate, respectively, were determined (Table I). In these determinations the Parnas and Wagner (21) micro-Kjeldahl method was employed, all determinations being carried out in duplicate and an average figure recorded.

A typical experiment is herewith given in detail:

11,500 cc. of 16 hour lysate was filtered in three equal portions (for the purpose of efficiency in filtration) through three separate Berkefeld filters. The combined filtrates were concentrated on a 5 per cent membrane and then washed with distilled water until the ultrafiltrate gave a negative test for ammonia. The concentrate remaining in the thimble (70 cc.) was collected and the nitrogen content and phage activity of each fraction were measured (Table I).

TABLE I

Details of Purification and Concentration of B. coli Phage

	Volume	Phage titre per cc.	N/cc.	N per unit of activity
	cc.		*mg.*	*mg.*
Crude phage	11,500	5×10^9	1.274	2.55×10^{-10}
Berkefeld filtrate	11,500	5×10^9	1.267	2.53×10^{-10}
Ultrafiltrate	11,600	No activity	1.256	No activity
Purified concentrate	70	10^{12}	0.018	1.8×10^{-14}

Comparison of the nitrogen content per unit of activity in the original lysate (about 2×10^{-10} mg. per unit) with that of the washed concentrate (about 1×10^{-14} mg. per unit) shows that by this simple procedure a considerable degree of purification has been attained inasmuch as the concentrate possesses all the original activity of the crude phage while the nitrogen content per unit of activity was reduced approximately 20,000 times.

Since, as stated earlier, most of the nitrogen in the crude phage probably consists of unassimilated ammonium succinate, while some indubitably represents bacterial debris, it was of interest to determine the ammonia and non-ammonia fractions of a Berkefeld filtrate in order to estimate how much of synthesized bacterial protein is actually removed during the process of purification. To determine this, the ammonia in a sample of crude phage was measured by aeration into standard acid and the total nitrogen by micro-Kjeldahl; the difference between the two values was taken as representing synthesized nitrogen. As a control on the efficiency of recovery of ammonia, an equal volume (1 liter) of sterile medium from the same batch was similarly aerated. The results of three such experiments are given in Table II.

TABLE II

Proportions of Ammonia and Non-Ammonia Nitrogen in Crude Phage in Synthetic Medium

Exp. No.	Bacteriophage			Sterile medium (control)		
	Total N	Ammonia N	Difference* (synthesized nitrogen)	Total N	Ammonia N	Difference (experimental error)
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	1329	1288	41	1341	1336	5
II	1273	1264	9	1280	1279	1
III	1269	1252	17	1294	1292	2
	Average.		22	Average. . . .		. . .3

* The wide variations in synthesized nitrogen are probably due to the fact that growth of bacteria prior to onset of lysis might have proceeded further in some of the experiments, thus permitting the assimilation of different amounts of ammonia.

It is seen that on an average, approximately 22 mg. of non-ammonia (synthesized) nitrogen were usually present in a liter of crude phage. Since the control determinations on the sterile synthetic medium showed that all but about 3 mg. of ammonia was recovered, this amount represents the probable experimental error. Assuming that the amount of synthesized nitrogen in the crude phage approximates on an average 20 mg. per liter, and knowing that it contains approximately 5×10^{12} active lytic units per

liter, it is evident that the crude lytic filtrate contains ($20 \div 5 \times 10^{12}$) 4×10^{-12} mg. of synthesized nitrogen per active unit. Since the purified phage has been found to have but 1×10^{-14} mg. N per unit, therefore, the phage represents about 1/400 of the total synthesized non-ammonia nitrogen of crude phage. The remainder must represent bacterial debris.

In order to ascertain the validity of these calculations it was of interest to determine how much of the nitrogen present in the original material was accounted for in the process of purification.

10,000 cc. of 16 hour lysate was filtered through three Berkefeld filters, collected on a 5 per cent membrane, washed until free of ammonia, and concentrated to 33 cc. The résumé of the distribution of nitrogen is given in Table III.

TABLE III

Completeness of the Recovery of Nitrogen during Purification

Crude phage	13,020 mg. N
Berkefeld filtrate	12,670 "
Removed by Berkefeld filter	350 "
Concentrate	0.825 mg N
Ultrafiltrate	12,640.000 "
Wash water	14.000 "
Accounted for	12,654.825 "
Berkefeld filtrate	12,670.000 mg. N
Accounted for	12,654.825 "
Unaccounted for	15.175 " N

While the procedure consisting of ultrafiltration is quite simple, it is apparently quite effective: practically all the activity (Table I), and practically all the nitrogen (Table III) of the original lysate can be recovered and it yields fairly reproducible results as can be seen from Table IV, which summarizes the results of a number of purification experiments carried out at different times over a period of about a year. From Table IV it can be seen that the average purified concentrate had a phage titre of the order of magnitude of 5×10^{12} lytic units per cc. and a nitrogen content of about 10^{-14} mg. per each lytic unit.

In order to secure a sufficient amount of material for chemical tests, and to calculate the weight of material per lytic unit, a number of purified preparations were reduced to dryness (the product being totally devoid of lytic activity). The results of seven such experiments are given in Table V.

Examination of Table V shows considerable differences in the dry weight per unit of activity and in the per cent nitrogen of different preparations. These differences are probably not as significant as they appear on first examination and probably represent the experimental errors involved in

TABLE IV

Summary of Purification Experiments with B. coli Phage

Original volume	Concentrate volume	Phage titre per cc.	N/unit of lytic activity
cc.	*cc.*		*mg.*
8,000	33.5	10^{12}	8×10^{-15}
11,500	70.0	10^{12}	1.8×10^{-14}
9,000	87.0	10^{12}	1.2×10^{-14}
10,000	33.0	3.5×10^{12}	7×10^{-15}
6,000	47.2	5×10^{11}	1.6×10^{-14}
6,000	45.2	6×10^{11}	1.5×10^{-14}
22,000	21.4	8.5×10^{12}	1.4×10^{-14}
20,000	26.3	5.0×10^{12}	1.5×10^{-14}
20,000	32.4	5.0×10^{12}	1.7×10^{-14}
18,000	17.3	8.0×10^{12}	1.4×10^{-14}
18,000	24.0	6.0×10^{12}	9.0×10^{-15}
18,000	25.0	5.0×10^{12}	1.2×10^{-14}
18,000	26.0	8.0×10^{12}	8.0×10^{-15}
14,000	39.0	2.0×10^{12}	1.2×10^{-14}

TABLE V

Nitrogen Content of Dried Purified Coliphage

Exp. No.	Original volume	Concentrate volume	Phage titre per cc. (conc.)	N/unit	Weight of dry residue	Dry weight/unit	Nitrogen of dry residue
	cc.	*cc.*		*mg.*	*mg.*	*mg.*	*per cent*
I	16,000	37.7	3.5×10^{12}	1.14×10^{-14}	9.58	7.26×10^{-14}	15.7
II	14,500	25.8	2.5×10^{12}	3.0×10^{-14}	8.88	1.4×10^{-13}	21.4
III	12,000	34.0	1.7×10^{12}	1.6×10^{-14}	5.27	9.1×10^{-14}	17.5
IV	8,000	43.0	7.0×10^{11}	2.0×10^{-14}	4.03	1.3×10^{-13}	15.4
V	8,000	33.0	9.0×10^{11}	2.0×10^{-14}	4.26	1.44×10^{-13}	13.9
VI	18,000	12.5	1.1×10^{13}	Average VI	6.98	5.07×10^{-14}	Average VI
VII	18,000	11.0	9.5×10^{12}	and VII =	6.70	6.41×10^{-14}	and VII
				7.8×10^{-15}			= 14.00

weighing of small samples, and inadequate washing of some of the residues. In round numbers these results indicate that each active lytic unit corresponds to approximately 6×10^{-14} mg. of dry residue containing 14–16 per cent of nitrogen.

This nitrogen content suggests the likelihood that the substance is a pro-

tein. In order to confirm this, protein color tests were carried out upon the dried material suspended in physiological saline. With suspensions containing about 2 mg. of dry residue per cc. it was possible to obtain positive biuret, Hopkins-Cole, and xanthoproteic reactions. The material contained sulfur and coagulated upon contact with strong acid. It is to be noted especially that this material contained no reducing sugars, even after hydrolysis with normal HCl at 100°C. for from one to several hours, in contrast to our own findings in 1927 and to the results of Northrop (11) who found about 1 per cent of glucose in his purified phage preparations.

In order to see whether the protein that has been isolated represents a specific substance synthesized only in the presence of bacteriophage, a similar procedure of purification and concentration was carried out upon filtrates of cultures grown on synthetic medium in the absence of bacteriophage.

B. coli P.C. was inoculated into 10 liters of synthetic medium and grown at 37° C. for 1 week in order to permit some autolysis to take place. This culture was filtered in equal portions through four Berkefeld filters, the filtrates were combined, concentrated, and washed upon a 5 per cent membrane exactly as were phage lysates. The last few cubic centimeters remaining on the membrane after copious washing and swabbing represented a water-clear solution which was divided into two portions and the total nitrogen of each determined. The results of three such experiments are given in Table VI.

TABLE VI

Nitrogen Content of Washed Concentrated Berkefeld Filtrates of B. coli P.C. Grown on Synthetic Medium in the Absence of Phage

Experiment No.	Original volume	Concentrate volume*	Average nitrogen content of ½ of sample†
	cc.	*cc.*	*mg.*
I	10,000	34.0	−0.0005
II	10,000	18.3	0.02
III	10,000	56.0	−0.001

* Since nitrogen content of the entire sample was determined, no attempt was made to keep the volume of concentrate constant.

† The apparently anomalous negative nitrogen value is an expression of the experimental error in nitrogen measurement.

These results show that the membrane which was used to collect bacteriophage in the purification procedure failed to retain any nitrogenous material from the filtrates of cultures of *B. coli* grown in the absence of bacteriophage, thus suggesting that the protein previously isolated is different from the protein present in autolyzed cultures of *B. coli*.

Properties of the Purified Phage

Activity and Stability

While the various preparations of purified bacteriophage differed somewhat in their activity, the greater number of concentrates gave a titre of about 5×10^{12} active units per cc., with a nitrogen content of about 10^{-14} mg. per unit of lytic activity.

In contrast to the marked stability of crude bacteriophage, which retains its activity for months even when reduced to dryness, the purified phage is very labile, losing its activity very rapidly even at ice box temperature and becoming completely inactive in 3 or 4 days. Addition of an equal volume of broth to a concentrate preserved the activity somewhat, but usually none was demonstrable beyond a week. If the preparation was dried or mixed with broth and then dried, the dry substance was always completely inactive.

The purified concentrated phage is antigenic, giving rise to antilytic antibodies upon parenteral injection into rabbits and guinea pigs. Formolized phage retains its antigenicity. However, if the phage is inactivated by drying, even in the presence of broth, as much as a total of 2.50 mg. of the phage protein injected in divided doses every other day for a total of four injections into each of six guinea pigs failed to stimulate the production of antilytic antibodies, while 1.80 mg. of a similar preparation, but not subjected to drying, injected into each of six guinea pigs was antigenic in every instance.

Chemical Properties

Two separate batches of 18 liters of phage each (representing a total of 2.32×10^{14} active lytic units) were purified and then dried in a vacuum desiccator. The dry weight of the combined residues was 13.1 mg. Some of this material (12.1 mg.) was analyzed through the kindness of Doctor A. Elek of The Rockefeller Institute, whose report was as follows:

The sample was dried *in vacuo* at 80°C. to constant weight. A sample of 3.092 mg. yielded 5.591 mg. CO_2, 2.182 mg. H_2O, and 0.030 mg. of ash, which corresponds to C = 49.31 per cent; H = 7.89 per cent; and ash = 0.97 per cent. A second sample of 3.602 mg. gave 0.442 cc. of N_2 at 25°C. and 757 mm., which corresponds to N = 14.00 per cent. A third sample of 3.580 mg. gave 0.195 mg. ammonium phosphomolybdate, which corresponds to P = 0.07 per cent.[4]

[4] It is of interest to note that the phosphorus content of our purified preparations is quite low (0.07 per cent) while Schlesinger (13), who also worked with a coliphage,

The material present in the bacteriophage concentrate is, therefore, apparently a protein. It contains 14–16 per cent nitrogen, gives positive protein color tests, and contains some sulfur. The extremely low phosphorus content of the sample analyzed by Doctor Elek (0.07 per cent) indicates that the substance as a whole is not a nucleoprotein, the phosphorus probably represents a trace of the phosphate from the medium which was not removed by washing.

Calculation of Molecular Weight

If a unit of lytic activity is assumed to be represented by one molecule of substance, the average molecular weight may be calculated from the weight of the particle by Avogadro's number. In the average preparation one unit of lytic activity was found to correspond to 10^{-14} mg. nitrogen (Table IV) or 6×10^{-17} gm. of dry residue (Table V) and, therefore, the gram molecular weight is of the order of magnitude of 36,000,000. The best preparation (7×10^{-15} mg. nitrogen per unit of activity), calculated as protein, would correspond to a molecular weight of 24,000,000.

Calculation of Particle Radius

With the average weight of substance corresponding to a unit of activity known (Table V), and assuming that each active unit represents: (1) one particle, (2) of spherical shape, and (3) of density 1.3,[5] the radius of the particle may be calculated:

Weight of particle (grams) $= \frac{4}{3}$ pi $R^3 \times 1.3$

Then:

$$R^3 = \frac{\text{Weight of particle in grams}}{\frac{4}{3} \cdot \text{pi} \cdot 1.3}$$

analyzing a sample of about 4 mg. (approximately the same size as ours) found 3.7 per cent phosphorus, and concluded that he had isolated a nucleoprotein. Northrop (11), working with a staphylococcus phage, likewise found a high phosphorus content (4.8 per cent) and similarly considered it a nucleoprotein. However, the fact that our purified preparation contained only 1 per cent of ash is in itself a confirmation of the low phosphorus content, since in our earlier preparations (1928–1931), which were less pure, Doctor Elek found 5–8 per cent phosphorus and a correspondingly high ash content (12–30 per cent) in the different preparations.

[5] The density of the bacteriophage particle has been estimated by various workers to be from 1.14 to 1.3 (11, 22–24). Since both Northrop (11) and Schlesinger (13) assumed a density of 1.3 in calculating particle radius, we have likewise assumed this value to facilitate the comparison.

Substituting in this formula the weight of a particle of an average preparation containing 6×10^{-17} gm. of dry residue per lytic unit (Table V), the calculated radius would approximate 22 millimicra, and on the basis of data for the best preparation (calculated as protein, 4×10^{-17} gm. per lytic unit) the particles would have a radius of 19 millimicra.

Attempts at Purification of Bacteriophage from a Yeast Medium

As has been mentioned earlier, Northrop (11) has been able to purify a staphylococcus bacteriophage propagated on yeast medium by a procedure which was essentially the same as that used by him for the purification of enzymes. Because the procedure is rather complicated, it is not always successful. However, during the past year Mr. Gordon Moore in this laboratory was successful in six out of ten attempts in recovering on an average 22.5 per cent of the total activity of the original bacteriophage (the highest activity being 42 per cent in one instance) by the procedure of Northrop (11).

The fact that we have been able to recover practically 100 per cent of lytic activity by a simple ultrafiltration may be to a large extent due to the fact that the bacteriophage we used was propagated on a simple completely diffusible synthetic medium. It was of interest, therefore, to see how effective our procedure might be in purifying a bacteriophage propagated on Northrop's yeast medium.

Since this medium probably contained some diffusible matter, and since the process of purification consisted in collection of the active agent on a collodion membrane, it was essential to remove this non-diffusible material which otherwise would be retained on the membrane together with the phage. For this reason all the yeast extract medium used in these experiments was ultrafiltered through a 5 per cent collodion membrane and autoclaved before being seeded with the bacteria to propagate the phage. The same coliphage was propagated on the *B. coli* P.C. grown on the ultrafiltered yeast medium and purified by collection and washing on a 5 per cent collodion membrane.

The technique in these experiments was exactly the same as has been previously described except that all ultrafiltrations were carried out in the ice chest to minimize contamination of the relatively rich yeast medium. In each case, as a control, a liter of sterile ultrafiltered medium from the same batch was similarly concentrated and washed and the amount of non-diffusible nitrogenous material determined (Table VII). Since there was no means of judging when the washing was completed, the concentrates collected on the membrane were arbitrarily washed with 2 liters of sterile distilled water. The results of one of three such experiments with *B. coli* are given in Table VII.

TABLE VII

Concentration and Partial Purification of Coliphage from Yeast Medium

	Sterile medium (control)	Bacteriophage	Corrected values
Original volume, *cc*	1,000	1,000	—
Concentrate volume, *cc*	15.2	21.9	—
Phage titre/cc	—	10^9	—
Dry weight/cc., *mg*	0.5	1.0	—
Total nitrogen, *mg*	0.26	1.05	0.79
Total dry weight, *mg*	7.6	21.9	14.3
Nitrogen/unit activity, *mg*	—	4.8×10^{-11}	3.6×10^{-11}
Dry weight/unit activity, *mg*	—	1.0×10^{-9}	3.5×10^{-10}
Nitrogen of dry residue, *per cent*	3.4	4.8	5 5

These results show that both the nitrogen content and dry weight per unit of activity of phage propagated on the yeast medium (4.8×10^{-11} and 1.0×10^{-9} mg., respectively) are significantly greater than the values obtained when bacteriophage is purified from synthetic medium (1×10^{-14} mg. nitrogen and 6×10^{-14} mg. dry residue per unit of lytic activity) (Tables I, IV, and V).

Although the yeast medium was subjected to ultrafiltration prior to seeding with the bacteria, the possibility that some of the nitrogenous constituents might undergo aggregation or coagulation during the subsequent autoclaving and thus be retained on the membrane may explain why some material was recovered when the liter of sterile ultrafiltered medium was concentrated as a control. However, even when the findings for phage (Table VII, column 2) are corrected by subtracting the values obtained from the sterile medium (Table VII, column 1), the nitrogen per unit of activity (3.6×10^{-11} mg.), the dry weight per unit of activity (3.5×10^{-10} mg.), and the total yield (14.3 mg.) are still far greater (Table VII, column 3) than those obtained when the same phage is purified from synthetic medium (Tables IV and V). Furthermore, the phage recovered from the yeast medium is apparently contaminated with a large amount of some non-nitrogenous material, since the per cent nitrogen of the dry residue is quite low (5.5 per cent). These results indicate that when yeast medium is used for the propagation of coliphage, ultrafiltration is not an efficient means for the purification of such preparations.

Determination of Particle Size of Bacteriophage

Earlier in this study, on the basis of the experimentally determined weight of protein per unit of lytic activity, the radius of the active particles has been calculated to be from 19 to 22 millimicra. This figure certainly repre-

sents a maximum average size of particle rather than a true average, since the preparation, while considerably purified, most likely still contained some extraneous material. Other investigators have made attempts to determine directly the particle size of bacteriophage by such methods as ultrafiltration through collodion membranes of known porosity (25-27), optical methods (28–29), centrifugation (11, 22–23), and rate of adsorption of phage by susceptible bacteria (30). These authors have all either assumed or concluded that a bacteriophage suspension is homogeneous; that is, that all the particles in a given strain are of the same size. However, other workers, on the basis of the difference in diffusion rates of various fractions of a given phage (5), and on the basis of an observed difference in the rate of diffusion of phage in a concentrated as contrasted with a dilute suspension (11), have concluded that any one phage must exist as a mixture of particles of widely different sizes.

Since the latter method is apparently more efficient in that it permits the detection of definite differences in particle size, it was adopted in these experiments. The technique of Hetler and Bronfenbrenner (15), who first applied to bacteriophage the method originally described by Northrop and Anson (31) for the determination of the particle size of carbon monoxide hemoglobin, has been employed in this study.

The procedure consists in measuring the rate of diffusion of bacteriophage through a porous membrane whose porosity has been standardized against a substance with known diffusion rate such as HCl.

The diffusion coefficient "D" is defined as the quantity of substance that will diffuse across a permeable plane of unit dimensions, in unit time, under unit concentration gradient. Northrop and Anson (31) expressed it mathematically, thus:

$$D = \frac{KQ_{\infty}.\ \text{cm.}^2}{t\ \text{day}}, \tag{1}$$

in which

D = diffusion coefficient.
K = diffusion constant of cell.
Q = cc. of substance diffused, expressed as a fraction of the original concentration.
t = time in days.

The relation between the diffusion coefficient, and the radius of the molecule, has likewise been derived as follows:

If water is used as a solvent, at 5°C.

$$r = \frac{1.148 \times 10^{-8}\ \text{cm.}}{D} \tag{2}$$

Three diffusion cells were standardized by measuring the rate of diffusion through them of HCl. The standardizations, as well as all further diffusion experiments, were carried out in the ice chest at 5° C., and the three cells were found to have the following constants (K): Cell I, 0.1736; Cell II, 0.1580; and Cell III, 0.1367.

The bacteriophage preparations used for measuring the diffusion rate were highly diluted so that the final titre was of the same order of magnitude (10^4 to 10^5 active lytic units per cc.) as that of the ultrafiltrates tested later (Table XI). The bacteriophage titre of the test solutions was measured by the McCrady (18) method previously described. In the experimental determination of the diffusion rate of phage, the cells were filled with a diluted phage and dipped into 30 cc. of the medium of dilution. Sixteen consecutive samples of the diffusates from each cell were secured at 30 minute intervals. The first four samples were discarded, and the remaining twelve were titrated for their phage content. The average of the twelve titrations gave the average number of units of lytic activity diffusing in 30 minutes. Since the number of lytic units diffusing through the porous disc in 30 minutes was rather small, a special method of titrating the activity (15) was used instead of the usual method of tenfold serial dilution. The 30 cc. sample of diffusate was thoroughly mixed and then added in amounts varying from 0.1 to 1.0 cc. to tubes of broth. Each tube was seeded with a drop of 1:10 dilution of an 18-24 hour broth culture of *B. coli*, incubated at 37° C., and examined for evidence of lysis the next day. From each tube which showed no evidence of lysis, one drop was deposited on a slant freshly seeded with *B. coli* P.C., incubated at 37° C., and examined for phage action 24 hours later. If no lysis was visible, it was considered that no phage was present. The concentration of phage in the test solution within the cell was so adjusted (10^4–10^5 units per cc.) that the number of units diffusing in 30 minutes could successfully be titrated by this method; *i.e.*, the end point of the titration (the volume representing one unit of phage) was between 1.0 and 0.1 cc. of diffusate.

Each diffusion experiment with a given phage preparation was carried out twice, using three cells simultaneously for each determination, thus resulting in six determinations on each test suspension. Below is described a typical diffusion experiment, employing as test solution a Berkefeld filtrate of *B. coli* phage in synthetic medium, diluted in synthetic medium. The titrations of the test suspension in duplicate are shown in Table VIII.

TABLE VIII

Titration in Duplicate of a Test Suspension of Coliphage in Synthetic Medium

Tube No	1	2	3	4	5	6	7	Control
Amount of original diffusate, *cc*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	0
Titration A	x*	x	5†	3	4	1	0	0
Titration B	x	x	5	4	2	1	0	0

* Dilution not tested.

† The number refers to the number of tubes (of the total of 5) showing lysis.

From the significant numbers (341 for titration A; and 421 for titration B), according to the probability tables (18), 10^{-4} cc. of the lysate contained

an average of 2.5 active lytic units, and its titre was therefore 25,000 active units per cc.

The details of the titration of the diffusates of one of the cells (cell I) are given in Table IX.

TABLE IX

Details of the Titration of Diffusates of Cell I

No. of tube..	1	2	3	4	5	6	7	8	9	10	Result
Amount of diffusate, *cc.*.....	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	Volume of diffusate containing 1 unit phage
Time of sampling *hrs.*											*cc.*
2.5	+*	+	+	+	+	−	−	−	−	−	0.6
3.0	+	+	+	+	+	−	−	−	−	−	0.6
3.5	+	+	+	+	−	−	−	−	−	−	0.7
4.0	+	+	+	+	+	−	−	−	−	−	0.6
4.5	+	+	+	−	−	−	−	−	−	−	0.8
5.0	+	+	+	−	−	−	−	−	−	−	0.8
5.5	+	+	+	+	−	−	−	−	−	−	0.7
6.0	+	+	+	−	−	−	−	−	−	−	0.8
6.5	+	+	+	+	−	−	−	−	−	−	0.7
7.0	+	+	+	+	−	−	−	−	−	−	0.7
7.5	+	+	+	+	+	−	−	−	−	−	0.6
8.0	+	+	+	−	−	−	−	−	−	−	0.8
Average											0.70

* + = lysis.
− = no lysis.

Since the total volume of diffusate in the beaker was 30 cc., and on an average 0.70 cc. contained 1 unit of phage (Table IX), it is clear that 30 ÷ 0.70, or 43 active lytic units, diffused through the disc in 30 minutes. Upon the assumption that each lytic unit represented one phage particle, the diffusion rate and the particle radius may be calculated. The fraction diffused (Q) was 43 ÷ 25,000 (original concentration of the test solution), or $Q = 0.00172$. From these data, knowing the cell constant (K), the diffusion rate may be calculated by means of equation 1.

$$D = \frac{KQ_{cc.}\ \text{cm.}^2}{t\ \text{day}}$$

$$= \frac{0.1736 \times 0.00172\ \text{cm.}^2}{0.0208}$$

$$= 0.01436\ \text{cm.}^2\ \text{per day.}$$

From the above diffusion rate (D), the average radius (r) of the particles is calculated by means of equation 2 to be equal to 8 millimicra as follows:

$$r = \frac{1.148 \times 10^{-6} \text{ cm.}}{D}$$

$$= \frac{1.148 \times 10^{-6} \text{ cm.}}{0.01436}$$

$$= 79.9 \times 10^{-8} \text{ cm.}$$

$$= 8.0 \text{ m}\mu.$$

By this procedure the rate of diffusion of both crude and purified phage was then measured. The test solutions were prepared in the following manner:

(A) *Crude Coliphage.*—*B. coli* P.C. phage was propagated in synthetic medium and filtered through a Berkefeld filter. The filtrate was diluted in sterile synthetic medium to give a phage titre of 10^4 to 10^5 active units per cc. This suspension was used as the test solution. (The results are given in Table X-A.)

(B) *Purified Coliphage.*—*B. coli* P.C. phage propagated in synthetic medium was concentrated on a collodion membrane, and purified by washing as previously described. This concentrate was diluted in synthetic medium to give a titre of 10^4 to 10^5 lytic units per cc. and this diluted suspension was used as the test solution (Table X-B).

It was of interest to compare the diffusion rate of coliphage with that of the staphylococcus phage (K) used by Northrop (11), but since the medium of suspension was different (staphylococcus will not grow on the synthetic medium) it was first necessary to measure the rate of diffusion of the coliphage when suspended in the yeast medium to see if the nature of the suspending medium had any significant effect on the rate of diffusion. These test suspensions were prepared as follows:

(C) *Crude Coliphage in Yeast Medium.*—*B. coli* P.C. phage was propagated in yeast medium, filtered through a Berkefeld filter, and then diluted with sterile yeast medium to give a test suspension with the same phage concentration as that used when phage in synthetic medium was employed (10^4 to 10^5 lytic units per cc.) (Table X-C).

(D) *Crude Staphylococcus K Phage in Yeast Medium.*—This test suspension was prepared exactly as was the crude coliphage in yeast medium (Table X-D).

The results of these experiments show that the average radius of the more rapidly diffusing phage particles in crude coliphage in synthetic me-

TABLE X

Diffusion Rates of Crude Bacteriophage Suspensions and Purified Bacteriophage

Test solution	Cell No.	Cell constant (K)	Total units phage in 30 cc. diffusate	Units phage in 1 cc. test solution	Fraction of phage diffused $\frac{\text{col. 4}}{\text{col. 5}}$ (Q)	Time in days (t)	Diffusion rate eq. (1) (D)	Average diffusion rate (Avg. D)	Average radius of particle in milli-micra eq. (2). (r)
									mμ
A	I	0.1736	111	62,500	0.00177	0.0208	0.01477		
	II	0.1580	120	62,500	0.00192	0.0208	0.01458		
	III	0.1367	150	62,500	0.00240	0.0208	0.01577	0.01461	7.9
	I	0.1736	43	25,000	0.00172	0.0208	0.01436		
	II	0.1580	46	25,000	0.00184	0.0208	0.01398		
	III	0.1367	54	25,000	0.00216	0.0208	0.01419		
B	I	0.1736	91	60,000	0.00152	0.0208	0.01269		
	II	0.1580	100	60,000	0.00167	0.0208	0.01268		
	III	0.1367	107	60,000	0.00178	0.0208	0.01170	0.01242	9.2
	I	0.1736	63	42,500	0.00148	0.0208	0.01235		
	II	0.1580	70	42,500	0.00165	0.0208	0.01253		
	III	0.1367	81	42,500	0.00191	0.0208	0.01255		
C	I	0.1736	63	35,000	0.00180	0.0208	0.01477		
	II	0.1580	65	35,000	0.00186	0.0208	0.01413		
	III	0.1367	73	35,000	0.00208	0.0208	0.01367	0.01443	7.9
	I	0.1736	86	50,000	0.00172	0.0208	0.01436		
	II	0.1580	94	50,000	0.00188	0.0208	0.01428		
	III	0.1367	115	50,000	0.00230	0.0208	0.01512		
D	I	0.1736	81	42,500	0.00191	0 0208	0.01594		
	II	0.1580	91	42,500	0.00214	0.0208	0.01626		
	III	0.1367	111	42,500	0.00261	0.0208	0.01715	0.01614	7.1
	I	0.1736	88	47,500	0.00185	0.0208	0.01544		
	II	0.1580	100	47,500	0.00211	0.0208	0.01603		
	III	0.1367	115	47,500	0.00244	0.0208	0.01604		

dium was about 8 mμ. By similar calculations, the radius of the particle of the purified phage was found to be about 9 mμ. This very slight difference in calculated radius is probably not significant; in view of the lability of the purified phage it is likely that some of the pure phage particles diffusing through are inactivated, thus leaving a smaller number of active particles in the diffusate, which results in a correspondingly higher calculated radius.

The character of the suspension medium apparently has no significant effect, since the coliphage propagated and diluted in yeast medium had exactly the same rate of diffusion and presumably the same particle size as that propagated and suspended in synthetic medium.

The average radius of the more diffusible particles of the staphylococcus phage appears to be somewhat smaller (7 mμ) but of the same order of magnitude as that of the coliphage.

However, as has been previously pointed out, Hetler and Bronfenbrenner (15) have demonstrated that, at least in the case of a coliphage propagated in broth, any one preparation may consist of particles of widely different sizes. They have suggested the possibility that the bacteriophage may represent a relatively simple chemical substance adsorbed on inert colloidal particles of different sizes present in the lysate. Thus, according to them, the size of particles calculated on the basis of diffusion rate is referred to the size of the vehicle particles rather than of the bacteriophage proper. More recently Northrop (11) has found that bacteriophage purified by him and presumably devoid of extraneous material also consisted of particles of different size depending on the degree of dilution. It, therefore, seemed of interest to ascertain by means of fractional ultrafiltration whether particles of different size are present at all times, independent of the dilution.

For this purpose, crude and purified coliphage and crude staphylococcus phage were fractionated by ultrafiltration through a thin collodion membrane, and the diffusion rate of the particles present in the ultrafiltrates (presumably the smallest particles) was measured. The details of the preparation of the test solutions are as follows:

(*a*) Crude *B. coli* P.C. phage in synthetic medium was forced through a 2 per cent collodion membrane and the ultrafiltrate, with a titre of about 10^4 active lytic units per cc. was used for the determination of diffusion rate (Table XI-*A*).

(*b*) Staphylococcus phage in yeast medium was similarly forced through a 2 per cent membrane and the ultrafiltrate which also had a titre of about 10^4 lytic units per cc. was likewise used as the test solution (Table XI-*B*).

(*c*) Enough concentrated purified *B. coli* P.C. phage, from synthetic medium, was added to dialyzed yeast medium[6] to give a titre of 10^9 to 10^{10} lytic units per cc. and this suspension was forced through a similar 2 per cent membrane, and the ultrafiltrate with a titre of about 10^4 units per cc. was used as the test solution (Table XI-*C*).

The average radius of the particles coming through the 2 per cent membranes was, as was to be expected, quite small, those from crude coliphage being about 1.3 mμ; those from purified coliphage about 1.9 mμ; and those from staphylococcus phage 2.4 mμ. Here again the differences between the results on crude and purified phage are probably not significant and might again be explained on the greater lability of the purified phage.

[6] The purified phage was suspended in yeast medium in order to preserve its activity as much as possible.

TABLE XI

Diffusion Rates of Ultrafiltered Crude and Purified Bacteriophage

1	2	3	4	5	6	7	8	9	10
Test solution	Cell No.	Cell constant (K)	Total units phage in 30 cc. diffusate	Units phage in 1 cc. test solution	Fraction of phage diffused $\frac{\text{col. 4}}{\text{col. 5}}$ (Q)	Time in days (t)	Diffusion rate eq. (1) (D)	Average diffusion rate (Avg. D)	Average radius of particle in millimicra eq (2). (r)
									mμ
A	I	0.1736	111	12,000	0.00925	0.0208	0.07720		
	II	0.1580	115	12,000	0.00958	0.0208	0.07277		
	III	0.1367	136	12,000	0.01133	0.0208	0.07441	0.08539	1.3
	I	0.1736	107	9,000	0.01189	0.0208	0.09924		
	II	0.1580	111	9,000	0.01233	0.0208	0.09366		
	III	0.1367	130	9,000	0.01444	0.0208	0.09490		
B	I	0.1736	42	8,750	0.00491	0.0208	0.04098		
	II	0.1580	52	8,750	0.00594	0.0208	0.04512		
	III	0.1367	75	8,750	0.00857	0.0208	0.05584	0.04708	2.4
	I	0.1736	42	8,000	0.00475	0.0208	0.03964		
	II	0.1580	50	8,000	0.00625	0.0208	0.04745		
	III	0.1367	65	8,000	0.00813	0.0208	0.05343		
C	I	0.1736	100	13,000	0.00769	0.0208	0.06418		
	II	0.1580	107	13,000	0.00823	0.0208	0.06252		
	III	0.1367	120	13,000	0.00923	0.0208	0.06066	0.06126	1.9
	I	0.1736	59	8,000	0.00738	0.0208	0.06159		
	II	0.1580	60	8,000	0.00750	0.0208	0.05697		
	III	0.1367	75	8,000	0.00938	0.0208	0.06165		

The results indicate that the dimensions of these particles (about 2 mμ in radius) are significantly different from those of the whole phage (about 8 mμ) and, therefore, demonstrate that the active particles in a given phage suspension vary widely in size.

Calculation of Molecular Weights

If the radius of the particle is known (calculated from the diffusion rate) and the density again assumed to be 1.3, the molecular weight may be calculated by the formula:

$$\text{Molecular weight} = \frac{4}{3}\,\text{pi}\,R^3N \times 1.3$$

where R is the radius of the particle in centimeters and N is Avogadro's number (6.06×10^{23} molecules in a gram molecular weight). The molecular weights of the crude and purified phages whose particle radii are given in Table X were calculated by this formula. For the crude coliphage with

radius of about 8 mμ, the calculated molecular weight would approximate 1,500,000; for the purified coliphage with radius of about 9 mμ, the calculated molecular weight is slightly higher, namely 2,250,000; and for the staphylococcus phage with radius of about 7 mμ, the molecular weight would be about 1,000,000. Similarly, the calculated molecular weights of the smaller particles which passed through the 2 per cent collodion membrane (Table XI) would be: crude coliphage—about 7,000; purified coliphage—about 22,000; and crude staphylococcus phage—about 45,000. The values for sizes and molecular weights of different preparations, calculated on the basis of diffusion rates are summed up in Table XII.

TABLE XII

Diffusion Rates, Calculated Radii, and Calculated Molecular Weights of Various Bacteriophage Preparations

Test solution	Average diffusion rate cm.²/day	Calculated average radius	Calculated average molecular weight
		mμ	
Crude coliphage . . .	0.01461	7 9	1,500,000
Purified coliphage. .	0.01253	9.2	2,250,000
Crude staphylococcus phage.	0.01715	7.1	1,000,000
Ultrafiltered (2 per cent collodion) crude coliphage	0.08539	1.3	7,000
Ultrafiltered (2 per cent collodion) crude staphylococcus phage	0.04708	2 4	45,000
Ultrafiltered (2 per cent collodion) purified coliphage	0.06126	1.9	22,000

These figures for radius and molecular weight (Table XII) are at best only approximations, since determination of a radius to a fraction of a millimicron is subject to a large experimental error. Since the molecular weights vary as the cube of the radii, a small difference in the size of the radius would give rise to a relatively large difference in calculated molecular weight. However, it can be seen from Table XI that the particles passing through the 2 per cent membrane, with radius of 1.2 to 2.4 mμ, and a calculated molecular weight of less than 50,000 are of an entirely different order of magnitude from those in the whole original phage suspension with radius of 7 to 9 mμ (Table X), and calculated molecular weight of some one to two million. These results, as stated earlier, indicate that active particles in any one phage differ in size so much as to indicate the simultaneous presence of particles of different orders of magnitude.

DISCUSSION

While earlier studies (1–10) have indicated that bacteriophage might be obtained free of protein, or at least in a form which failed to give protein

color tests, recent more exact studies (11–14) concerning the chemical nature of bacteriophage have indicated that this active agent represents a new type of substance, the so called "heavy protein" with a molecular weight of many millions, similar to those isolated from tissues of animals (infected with equine encephalomyelitis (32) and with virus-induced rabbit papilloma (33)), and plants (infected with tobacco mosaic virus (32) and with cucumber viruses 3 and 4 (34), with potato virus "X" (35), and with tomato bushy stunt virus (36)).

The present studies on the concentration and purification of a coliphage by means of ultrafiltration have resulted in the isolation of a substance containing approximately 15 per cent of nitrogen, giving positive color tests, and therefore presumably a protein, and retaining all the activity of the original lysate. In this product there was five to ten times less nitrogen per unit of lytic activity (10^{-14} mg.) than was found by Schlesinger (13) for a coliphage and by Northrop (11) for a staphylococcus phage. If each unit of lytic activity is assumed to represent one molecule of protein, the molecular weight calculated from the weight of dry residue per unit (6×10^{-17} gm.) would be approximately 36,000,000, and the calculated radius of the molecule about 20 millimicra. These dimensions correspond quite well with the heavy protein previously mentioned.

However, earlier studies (Hetler and Bronfenbrenner (15)) have demonstrated on the basis of the measurement of diffusion rate that bacteriophage may exist as particles with a radius of the order of magnitude of 1 millimicron and a calculated molecular weight below 100,000, which possess all the properties of the original active agent. These findings were confirmed in the present study. Measurement of the diffusion rate of particles of purified phage in dilute solution demonstrated the existence of particles with an average radius of about 9 millimicra corresponding to a molecular weight of about 2,250,000. Since the concentrated, purified phage may not be completely free of inert material, the dimensions calculated from the total weight of material per unit of activity probably represent the maximum limits of average size rather than a true average. On the other hand, calculation from the measurement of diffusion rate probably gives dimensions that are somewhat low, since any environmental influences that increase the motion of the particles would tend to give a higher rate of diffusion than actually exists, and consequently a lower calculated value for particle size. Furthermore, when diffusion rates are measured, employing a suspension containing particles of various sizes, the measured dimensions do not represent a true average, but the average of the particles which diffuse faster; *i.e.*, the smaller particles. Thus, calculations of the radius of

particle from weight give a value that is too high (20 millimicra), while those from measurement of the diffusion rate tend to give values that are too low (9 millimicra), and the true average size probably lies somewhere between these two values.

However, our studies indicate, as Hetler and Bronfenbrenner (15) have demonstrated earlier, that in addition to these larger particles there are present also active particles of an entirely different order of magnitude. If the purified phage is fractionated by ultrafiltration through a collodion membrane which permits the passage of only the smaller particles, and the average size of the particles in this ultrafiltrate is measured on the basis of their diffusion rate, the average radius of the particles diffusing in this case is found to be about 2 millimicra, corresponding to a molecular weight of about 25,000. This figure is significantly different from the one obtained using purified but unfractionated phage, and may be taken as indicating that the active agent is distributed as particles of widely different size. Since the smaller particles possess the characteristic activity of bacteriophage, it seems that in calculating molecular weight it is more appropriate to take as a basis the smallest particle exhibiting the lytic property of the agent. The larger particles must then be either aggregations of the smaller particles which dissociate on dilution, as suggested by Northrop (11), or they represent inert protein to which a small molecule of the active agent is adsorbed, as suggested by Bronfenbrenner (20). In either case, the molecular weight of the active agent should not be calculated on the basis of the size of the largest particles, but on the smallest ones. Our results suggest that the small particles exist at all times and do not necessarily appear as a result of dilution, since both sets of experiments (those with the whole phage and those with the ultrafiltered fraction) were carried out with relatively dilute suspensions, the purified phage having about 20,000–60,000 active units per cc., while the ultrafiltrate of the purified phage had about 9,000–13,000 active lytic units per cc. If, as Northrop suggests, the presence of smaller particles is due to depolymerization taking place on dilution, it would seem probable that this depolymerization would have occurred by the time the phage was diluted 100,000,000 times from its original concentration of about 5×10^{12} to about 5×10^{4} units per cc. present in the material used in determining the diffusion rates of the whole phage, and that no more depolymerization would be expected to take place on a relatively slight (fourfold) further dilution from 40,000 to 9,000 active units per cc. Therefore, it is unlikely that the large particles represent aggregates which may be dissociated by dilution (since the size obtained by simple dilution is not the same as that obtained by fractionation by

ultrafiltration), but rather that they may consist of inert colloidal protein carrier particles to which the smaller active molecules of phage have been adsorbed. The calculated molecular weight of these smaller particles of phage (about 25,000) is well within the order of magnitude of that of proteins but, of course, of much smaller size than the heavy proteins previously mentioned. Whether these smaller particles actually represent free molecules of the active agent cannot be decided at present, although from a consideration of their small size and low calculated molecular weight it is possible that they do represent such free molecules.

It is interesting to note in this connection that Topley and Wilson (37) (1937) consider the relative uniformity of size among active particles of a given strain of bacteriophage as evidence in favor of the microorganismal nature of the agent. While a discussion of the living nature of bacteriophage is outside of the scope of this study, it should be pointed out that our findings contradict the assumption as to uniformity of size of particles, although not necessarily the living nature of phage. It must be admitted, however, that while the results of this investigation contribute no direct evidence to a solution of the problem of whether phage is or is not a living organism, it is rather difficult to imagine a relatively simple substance of molecular weight of only about 25,000, which probably represents but one protein molecule, to be a living organism.

SUMMARY

A simple method of concentrating and purifying bacteriophage has been described. The procedure consisted essentially in collecting the active agent on a reinforced collodion membrane of a porosity that would just retain all the active agent and permit extraneous material to pass through. Advantage was taken of the fact that *B. coli* will proliferate and regenerate bacteriophage in a completely diffusible synthetic medium with ammonia as the only source of nitrogen, which permitted the purification of the bacteriophage by copious washing.

The material thus obtained was concentrated by suction and after thorough washing possessed all the activity of the original filtrate. It was labile, losing its activity in a few days on standing, and was quickly and completely inactivated upon drying. This material contained approximately 15 per cent of nitrogen and with 2 or 3 mg. samples of inactive dry residue it was possible to obtain positive protein color tests.

The concentrated and purified bacteriophage has about 10^{-14} mg. of nitrogen, or 6×10^{-17} gm. of protein per unit of lytic activity. Assuming that each unit of activity represents a molecule, the calculated maximum

average molecular weight would be approximately 36,000,000, and on the assumption of a spherical shape of particles and a density of 1.3, the calculated radius would be about 22 millimicra.

By measurement of the diffusion rate, the average radius of particle of the fraction of the purified bacteriophage which diffuses most readily through a porous plate was found to be of the order of magnitude of 9 millimicra, or of a calculated molecular weight of 2,250,000. Furthermore, when this purified bacteriophage was fractionated by forcing it through a thin collodion membrane, which permits the passage of only the smaller particles, it was possible to demonstrate in the ultrafiltrate active particles of about 2 millimicra in radius, and of a calculated molecular weight of 25,000.

It was of interest to apply this method of purification to a staphylococcus bacteriophage. Since this organism does not readily grow in synthetic medium, a diffusate of yeast extract medium was employed. The better of two preparations contained about 10^{-12} mg. of nitrogen per unit of lytic activity. Although this is about one hundred times the amount of nitrogen found in an active unit of *B. coli* bacteriophage, nevertheless, the diffusion rate experiments gave results which paralleled those obtained with the coliphage. The diffusible particles of the crude staphylococcus bacteriophage had a radius of about 7 millimicra, and a calculated molecular weight of about 1,000,000, while the particles of the same phage which appeared in the ultrafiltrate through a thin collodion membrane had a radius of about 2.4 millimicra and a calculated molecular weight of about 45,000.

It appears, therefore, that the active principle is distributed as particles of widely different sizes. However, since the smaller particles have all the properties of bacteriophage, the larger particles probably do not represent free molecules, but either are aggregates, or more likely, inactive colloids to which the active agent is adsorbed.

The protein isolated, which bears the phage activity, is capable of stimulating the production of antilytic antibodies on parenteral injection into rabbits or guinea pigs. It retains its specific antigenicity when inactivated by formalin, but not when inactivated by drying.

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THE FLICKER RESPONSE CONTOUR FOR THE FROG

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I

The determination of the flicker reponse contour for amphibians is of interest for several reasons. In typical frogs the proportion of retinal rods to cones is fairly high, though not so large as in some fishes or in man. The presence of "double" cones and certain other features of the retina point to the possibility of complications in the data of visual performance. A good deal of information about the excitability of the frog's eye and visual system is already available. In the newt *Triturus*[1] the curve of log critical intensity as a function of flash frequency has already been shown to possess some unusual aspects. We have made observations with several kinds of frogs and toads; homogeneous data have been obtained with *Rana pipiens*, defining what may be taken to be the typical form of the $F - \log I$ contour for these Anurans. We have not been successful in observing responses to flashing light suitable for analytical work with various tadpoles tested. The apparatus and the general procedure were the same as earlier described[2] in accounts of studies with a variety of other animals.

With various fishes, *Triturus*, and man, all possessing both rods and cones, the $F - \log I$ curve (band) shows a manifestly duplex constitution.[2,3] This is also found for *Rana*. The proportionate contributions of "rod" and "cone" elements to the composite curve, in relation to the numbers of rods and of cones, give information respecting certain aspects of the duplexity theory.

II

Rana pipiens from Vermont, about one year old or more, were used in the spring and early summer. These frogs had been collected during the previous summer and autumn and stored in cages in a pond. They were kept in the laboratory for about one week

[1] Data to be published elsewhere; *cf. Proc. Nat. Acad. Sc.*, 1938, **24**, 125.

[2] *J. Gen. Physiol.*, 1935–36, **19**, 495; 1936–37, **20**, 211, etc.

[3] *J. Gen. Physiol.*, 1936–37, **20**, 411; 1937–38, **21**, 17, 203, 313; 1938–39, **22**, 463, 487. *Proc. Nat. Acad. Sc.*, 1938, **24**, 125.

before the observations began. From a lot of twenty-five, six frogs were selected on the basis of small size and reactivity. These six were then kept individually in numbered jars on moist sphagnum, and fed at intervals with *Tenebrio* larvae. For the observations each frog was put into a small thin-glass crystallizing dish covered by a watch-glass held on with adhesive tape. These dishes fit inside the rotating striped cylinder in the apparatus. Tests were made after a minimum of 0.5 hour dark adaptation in a thermostat at 21.5°C. and with a flash cycle in which light intervals and dark intervals were equally long.

Data were secured at fixed flash frequencies (F) by increasing the intensity (I) of illumination until the threshold response was evidenced. The converse test,[4] made by keeping I constant and decreasing F from a high level until establishment of the relation between F and I critical for response to recognition of flicker, results with these frogs in either a failure to show recognizable response or in an erratic response at a very much lower F than tests of the reverse kind show to be appropriate. This is undoubtedly an effect of exposure to light.

With F fixed, increase of I from 0 or a very low level leads to a reaction which consists in a sudden straightening up of the body; this may or may not be accompanied by hopping in the direction of the moving stripes, or against this direction. In most cases only one such movement is made. Thereafter the frog remains in a state of tonic immobility. In some individuals there occurs at the threshold of response a moving of one or both front legs, and occasionally a jumping toward the moving stripes.

When the individual is in the state of tonic immobility we have referred to, subsequent to a threshold response, or following exposure to light in the apparatus, an attempt to repeat the reaction at threshold is fruitless. Even when the frog is touched or pushed to a new position hardly any response can be induced. Only a period of confinement to darkness or a decrease of F to less than 5/sec., even with the highest intensities of light, will bring the animal out of its non-reactive state. Only in exceptional cases are two or three successive responses obtainable without arousing the frog from immobility.

There seemed to be no detectable diurnal cycle of responsiveness in these frogs; but with increasing duration of residence in darkness at any time of day the reactiveness to flicker is notably decreased. This was the basis for the practice employed of not continuing observations beyond 1 or 2 hours confinement in darkness. The frogs were then returned to the light.

These difficulties of observation led as a practical matter to the restriction of the experiment to the use of six individuals. One of these was lost after eight sets of readings had been secured. In Table I, therefore, the values of I_m are for these eight sets based on eighteen observations each; for the other entries on fifteen; three readings were taken on each individual at each F. Table I contains three values of I_m based upon preliminary experiments; these are enclosed in parentheses, and in Fig. 1 they are distinguished by tags.

A variable analysis[5] of the individual measurements shows that the six (five) frogs of this lot were essentially equivalent. Their respective rank-order positions as regards excitability are distributed in an entirely random way.

[4] *J. Gen. Physiol.*, 1936–37, **20**, 211, 363; 1938–39, **22**, 311, 555.

[5] *Cf. J. Gen. Physiol.*, 1935–36, **19**, 503; 1936–37, **20**, 211, 363, 411; 1938–39, **22**, 311, etc.

III

Inspection of the relationship between I_m and F (Fig. 1) shows that log I_m as a function of F is in the main a sigmoid curve rising to a maximum F at a little above $F = 40$. At its lower end there is a comparatively small "swelling" on the tail of this curve. This corresponds to the rod part of

TABLE I

Log mean critical flash intensity (*millilamberts*) *vs.* flash frequency (F, per sec.); *Rana pipiens*; 21.5°C. Preliminary values in parentheses.

F	log I_m	log P.E.$_{.1}I_1$
2	$\bar{5}.3827$	$\bar{6}.1875$
3	$\bar{5}.6381$	$\bar{6}.1024$
4	$\bar{5}.8017$	$\bar{6}.2543$
5	$\bar{5}.9867$	$\bar{6}.8088$
	$\bar{5}.9868$	$\bar{6}.6639$
	$\bar{4}.0175$	$\bar{5}.1202$
7	$\bar{4}.3130$	$\bar{5}.0338$
8	$\bar{4}.6907$	$\bar{5}.2198$
9	$\bar{4}.9835$	$\bar{5}.8306$
10	$\bar{3}.4138$	$\bar{4}.4306$
	$\bar{3}.4197$	$\bar{4}.5480$
	$\bar{3}.3964$	$\bar{4}.7383$
15	$\bar{2}.1316$	$\bar{4}.9693$
	$\bar{2}.1644$	$\bar{3}.1402$
20	$\bar{2}.8095$	$\bar{3}.5935$
	$\bar{2}.7662$	$\bar{3}.4716$
	($\bar{1}.0141$)	($\bar{3}.6538$)
25	$\bar{1}.3551$	$\bar{2}.3877$
	$\bar{1}.3290$	$\bar{2}.3683$
	($\bar{1}.2019$)	($\bar{2}.4803$)
	$\bar{1}.3326$	$\bar{2}.1544$
30	0.0948	$\bar{2}.8577$
	0.0881	$\bar{2}.8054$
	(0.2828)	($\bar{1}.1714$)
35	1.0274	$\bar{1}.2403$
38	1.7116	0.6720
39	2.2325	0.4886

the dark-adapted flicker response contour in man, *Triturus*, and various fishes.[3] It does not correspond to the small distortion at the lower end of the $F - \log I$ curve for the gecko,[6] since it is not (as proved for the gecko) due to the wider opening of the iris for intensities below a certain level. The frog iris, in the intact animal, does not perceptibly alter its diameter

[6] *Proc. Nat. Acad. Sc.*, 1938, **24**, 538. *J. Gen. Physiol.*, 1938–39, **22**, 555.

as a function of illumination. It is moreover resistant to the action of instilled atropine or pilocarpine. We have made determinations of critical illumination at several flash frequencies under these treatments, but found

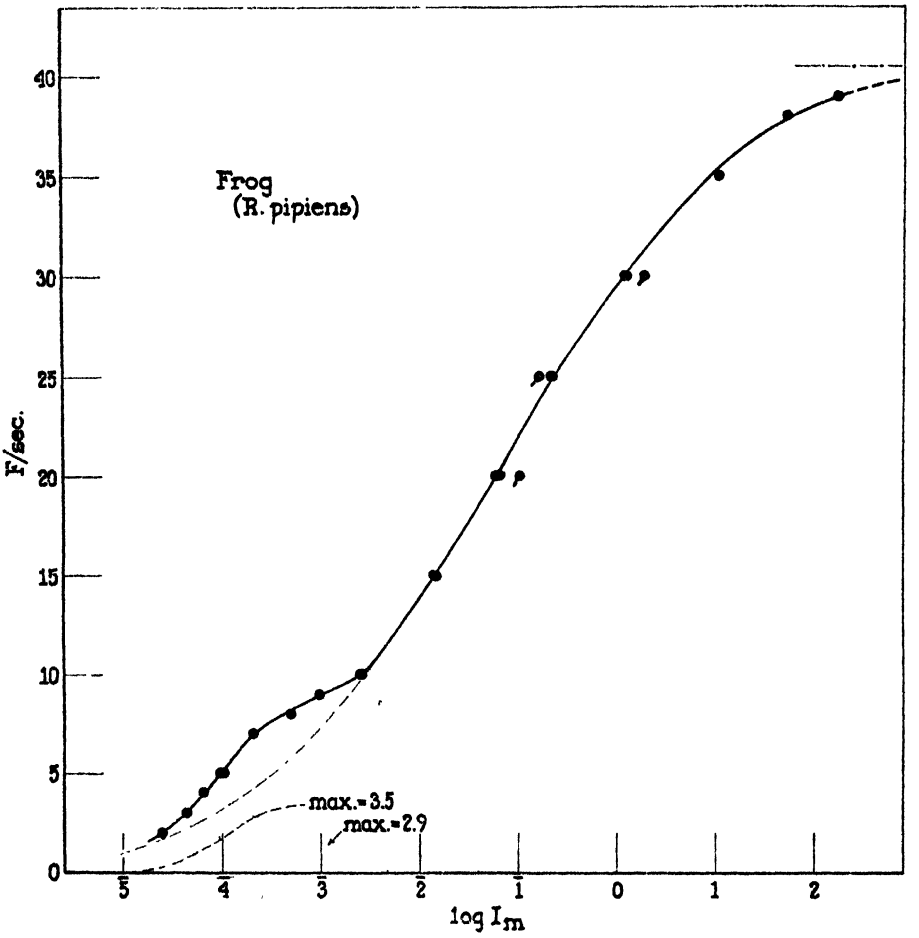

FIG. 1. Log mean critical intensity as a function of flash frequency for *Rana pipiens*. Data in Table I; points with tags are preliminary, as indicated in Table I. (The curves at the lower left are discussed in the text.)

no divergence from the normal. In addition the form of the curve at the low intensity end is not the same as in the gecko case.

As with the data on man and *Triturus* the upper, larger portion of the curve is very well described by a probability integral (Fig. 1) while the lower part cannot be directly so described in a satisfactory way. The lower,

"rod" part of the contour overlies the "cone" curve and its ordinates are added thereto. The separation of the rising and falling branches of the "rod" contribution by subtraction of the extrapolated probability integral for the "cone" part[7] is shown in Fig. 1. The rising and declining part of the "rod" curve as drawn are each probability integrals. The summation of these curves with that for the "cone" segment accurately reproduces the course of the data.

It has been pointed out[8] that the decline of the contribution to the curve derived from "rod" excitations can perhaps be understood as due to the (central) inhibition of rod effects in consequence of cone excitations. It is not inconsistent with this view that the maximum F values which have to be taken for the rising and the declining rod curves respectively (Fig. 1) are not the same; the rising branch is apparently cut off before it reaches its maximum level. It is recognized, of course, that the "rod" section of the frog curve is so diminutive that no emphasis should be placed upon minutiae of its analysis.

Unlike the curve for *Triturus*,[1] there is no indication in Fig. 1 of more than two groups of elements of effect revealed in the $F - \log I$ contour for the frog.

IV

In considering the variation of critical intensities as obtained in homogeneous measurements it has been found that I_m and the dispersion of I_1 are in simple, direct proportion for those cases in which only one class of elements or units of sensory effect are implicated. This is the case for arthropods,[9] even when mechanical conditions of peripheral photoreception bring about a distortion of the shape of the $F - \log I$ curve. It is also found for the turtle *Pseudemys*[10] and for the gecko *Sphaerodactylus*;[11] in these forms we have to do with only one general class of retinal elements (cones, rods, respectively). For vertebrates in general, exhibiting the typically duplex form of flicker response contour, a graph of $\log \text{P.E.}_{1f_1}$ *vs.* $\log I_m$ is found to exhibit a lower band of direct proportionality breaking at an intermediate intensity to a band of lower slope.[2] The intensity level at which this break appears is that at which the putative rod effects are no

[7] *J. Gen. Physiol.*, 1936–37, **20**, 411; 1937–38, **21**, 17, 203, 313; 1938–39, **22**, 463.

[8] *J. Gen. Physiol.*, 1938–39, **22**, 463.

[9] *J. Gen. Physiol.*, 1935–36, **19**, 503; 1936–37, **20**, 363, 393; 1937–38, **21**, 223, 463; 1938–39, **22**, 451, 795; 1939–40, **23**, 1.

[10] *J. Gen. Physiol.*, 1938–39, **22**, 311.

[11] *J. Gen. Physiol.*, 1938–39, **22**, 555.

longer involved.[12] The measurements at intensities higher than this level can be shown[13] to follow the law of direct proportionality between I_m and $\text{P.E.}_{.1I_1}$, but with an origin different from that for the zone of lower intensities. That is, for the purely cone part of the function we have $(I_m + \text{const.}) = k\,(\text{P.E.}_{.1I_1})$. In a number of the cases studied the transi-

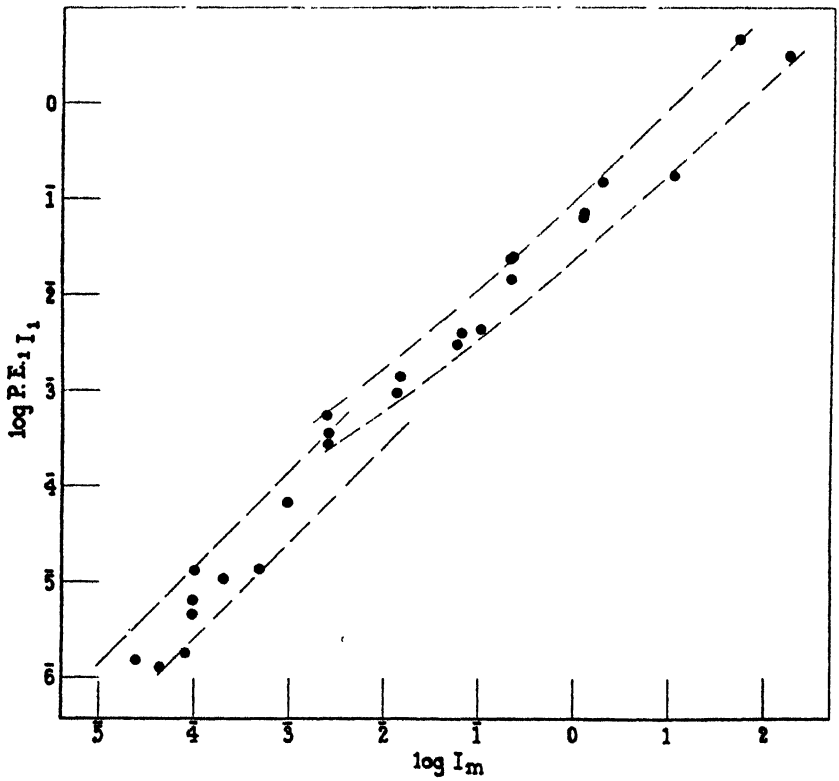

FIG. 2. Relation between log I_m and log $\text{P.E.}_{.1I_1}$ in Table I. Only for the lower part is the proportionality of I_m and $\text{P.E.}_{.1}$ simple and direct.

tion level, below which we have simply $I_m = k\ \text{P.E.}_{.1I_1}$, is at so high an intensity that only a small range of uncomplicated cone effects is available.[14] For the newt *Triturus*[1] the transition is at a comparatively low intensity. With *Rana* it occurs at an even lower intensity. Fig. 2 shows that below log $I = \bar{3}.4$ (or a little higher) we have log I_m and log $\text{P.E.}_{.1I_1}$ in direct

[12] *Cf. J. Gen. Physiol.*, 1937–38, **21**, 203; 1938–39, **22**, 463.
[13] *J. Gen. Physiol.*, 1935–36, **19**, 503.
[14] *J. Gen. Physiol.*, 1938–39, **22**, 463.

proportion with a slope of 1. Above this intensity a different relationship obtains. Fig. 3 shows that log $(I_m + 0.00646)$ and log P.E._{1I_1} are rectilinearly related with a slope of 1. It is scarcely to be taken as accident that log 0.00646 = $\bar{3}.81$ is precisely at the level (Fig. 1) at which the rod effects fade out completely.

One significant point to which we may again call attention in this connection is that if I_m and P.E._{1I_1} are directly proportional, so that the relative variation of I_1 is constant, we can say that $\text{P.E.}_{\log I_1}$ is constant. But for the uncomplicated cone portions of these flicker response contours

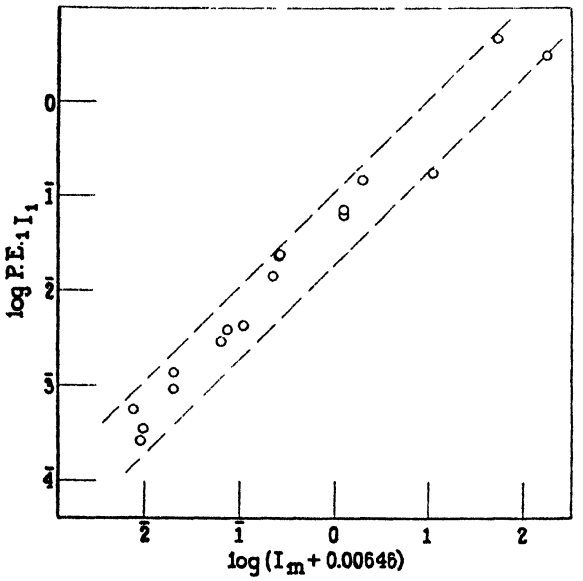

FIG. 3. In the higher intensity range P.E._{1I_1} is directly proportional to $(I_m + 0.00646)$. See text.

the relative variation of I is obviously not constant, hence $\text{P.E.}_{\log I_1}$ cannot be constant. This has a definite bearing upon the decision as to whether I or log I is the proper variable to employ as an index of excitability. For various reasons we have been led to the conclusion that while $dF/d\log I$ gives, as a function of log I, a frequency distribution of effects produced, the frequency distribution of momentary thresholds is given by $-dF/dI$.[15] These considerations indicate that the critical intensity, not its logarithm, is to be used for the analytical index of excitability; on this basis, for exam-

[15] *Cf. Proc. Nat. Acad. Sc.*, 1937, **23**, 71; 1939, **25**, 78, 171. *J. Gen. Physiol.*, 1937-38, **21**, 313, 463; 1938-39, **22**, 311, 487, 795.

ple, the behavior of the excitability as a function of temperature and of the light-time cycle fraction can be consistently accounted for.

V

Curves of visual performance as obtained from typical vertebrates exhibit two distinct parts or sections. Such animals are known to possess retinal rods and cones. It is shown in several different ways that these two segments must be considered to possess distinctive physical characteristics.[16] As has been emphasized, however, this merely allows us to conclude that we are dealing with two distinct groups of elements of sensory effect. We are not required, or even allowed, to deduce that these two groups represent the properties of rods and cones in any direct way. We have for one thing the demonstration that a gecko[11] with rod retina and turtle[10] with cone retina provide (simplex) performance curves almost identical with respect to form and position on the intensity axis.

The $F - \log I$ contour for *Rana* is quite similar to that for the newt *Triturus*,[1] save that in the former (as already noted) there is no indication of a third group of elements. The curve for the frog attains a slightly lower maximum F (*Rana*, 40.5/sec., *Triturus*, 43.7/sec.). The curves—at the same temperature 21.5°, and with 50 per cent light-time in the flash cycle—cross twice; at the high F end and also near $F = 7$. The abscissa of inflection ($\log I_{\text{inf.}}$) of the cone curve is lower for *Rana* ($\log I = \bar{2}.81$) than for the newt ($\log I = \bar{1}.72$), and $\sigma'_{\log I}$ is higher. The "rod part" is definitely smaller for the frog.

By a procedure differing from ours in certain essentials Birukow[17] obtained a flicker response curve for *Rana temporaria*. Under conditions of long light adaptation his curve is not duplex and rises to a flat maximum at $F = 6$. That under light adaptation the curve is depressed from that shown in Fig. 1 we can confirm. But we are also convinced that with prolonged light adaptation the state of tonic immobility to which we have referred (section II) makes it difficult to secure valid readings. On the other hand, again employing reactions to movement of a striped cylinder surrounding the animal, Birukow obtained data[18] on "visual acuity" for threshold response which provide an interesting parallel with our $F - \log I$ band in Fig. 1. These data (1/(*visual angle*) *vs.* $\log I$) show a slowly rising section up to about $\log I = \bar{2}.6$, followed by a steep rise up to about $\log I = 1.0$ (millilambert units). By means of tests with colored stripes Birukow[18] was able to satisfy himself that color vision is demonstrable for

[16] *J. Gen. Physiol.*, 1936–37, **21**, 411; 1937–38, **21**, 17, 313; 1938–39, **22**, 463, 487.
[17] Birukow, G., *Z. vergleich. Physiol.*, 1937, **25**, 92.
[18] Birukow, G., *Z. vergleich. Physiol.*, 1939, **27**, 41.

red and blue above 0.04 lux (= 0.0036 ml., log = $\bar{3}.23$), with a mixture of effects due to photopic and scotopic vision between 0.04 lux and 30 lux (*i.e.*, between log I (ml.) = $\bar{3}.23$ and 0.186). One cannot be certain of the physical significance of results based on the use of colored papers. The temperature is not given, so that although the level 0.04 lux is precisely in the "angle" (log I = $\bar{3}.23$) of our curve in Fig. 1 the exactness of the correspondence is possibly fortuitous.

The proportion of rods to cones is approximately 2.5:1 in the thickened[19] (? pseudofoveal) region of the frog retina, in more peripheral regions *ca.* 3.5:1.[17] Nevertheless the rod contributions to the $F - \log I$ curve (Fig. 1) are the smallest, absolutely, thus far recognized with any vertebrate exhibiting visual duplexity.[3] In a similar way the dark adaptation contour for the frog's eye (as determined from the progressing increase of electrical response) shows[20] a proportionately much enlarged "cone" segment by comparison with that for man (rods: cones = about 25:1). It is to be remembered, however, that in the present experiments a light/dark ratio of 1 was used, and that the rod and cone contributions may behave differently as a function of changes in this ratio, as found for *Enneacanthus*.[21] This alone would introduce complications in any attempt to correlate numbers of retinal elements with the response to flicker. In *Fundulus*, where the rod:cone ratio is *ca.* 2:1,[22] we find the "rod" part of the flicker response contour to be three times as great as in the frog.

SUMMARY

The flicker response contour for the frog *Rana pipiens* exhibits the duplex character typical for most vertebrates. By comparison (under the same conditions of temperature, 21.5°, and light-time fraction, = 0.5), the low intensity section of the $F - \log I$ curve is the smallest thus far found.

The cone portion of the curve is satisfactorily described by a probability integral. The rod part represents the addition of a small group of sensory effects upon the lower end of the cone curve, from which it can be analytically separated.

The relation between the two groups of sensory effects permits certain tests of the rule according to which (in homogeneous data) I_m and σ_{1I_1} are in direct proportion.

[19] Chievitz, J. H., *Arch. Anat. u. Physiol., Anat. Abt.*, 1891, 311.
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THE DENATURATION OF PROTEINS BY SYNTHETIC DETERGENTS AND BILE SALTS*

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All the synthetic detergents and bile salts I have tried denature proteins such as hemoglobin and egg albumin at the isoelectric point[1] and keep the denatured isoelectric protein in solution. Some detergents in sufficiently high concentration can prevent the precipitation of denatured protein by trichloracetic acid, tungstic acid, and acid ferric sulfate. In fact, the origin of the present investigation was the observation that a detergent used to clean test tubes containing hemoglobin precipitated by trichloracetic acid dissolved the precipitated hemoglobin.

The synthetic detergents and the bile salts all have the same general type of hydrophobic-hydrophilic structure. Each consists of a large hydrophobic part with a small hydrophilic part attached to it. The detergents are extremely surface active and their hydrophobic groups can combine with hydrophobic particles to give these particles a coating of hydrophilic groups.

Since the synthetic detergents and bile salts differ widely in their specific chemical structures, the denaturation of proteins and the solution of denatured protein by synthetic detergents and bile salts must be attributed to the one property these compounds have in common, their type of hydrophobic-hydrophilic structure.

There is a great variety of synthetic detergents available commercially partly because each type of detergent is patented and partly because by variation in the specific structures of the detergents, detergents have been developed which are particularly suited for specific industrial uses. Although the synthetic detergents were originally introduced as soap substitutes their industrial uses are now very varied. As soap substitutes and

* A brief account of denaturation by detergents was published in *Science* (Anson, 1939).

[1] The isoelectric point of a protein is necessarily changed as a result of combination of the protein with a detergent, to what extent is not yet known.

as protein reagents the synthetic detergents have the great advantage of being soluble in neutral and in most cases in acid solution.

The great differences between the specific structures of the different detergents are shown by a brief description of the detergents I have used. Unfortunately the manufacturers do not in all cases give complete chemical information about their products.

The Duponols have the general structure $CH_3(CH_2)_nCH_2OSO_3Na$. Duponol 80 is the sodium salt of the sulfuric acid ester of octyl alcohol. Duponol Special WA paste and its dry form, Duponol ME dry, consist mainly of the C_{12} compound which is called sodium dodecyl sulfate. Duponol PC is a dried mixture of the C_{10}–C_{18} compounds from which the unesterified free alcohol has been removed. The Duponols with less than eight carbon atoms are not effective detergents. The Duponols with very long fatty acid chains are insoluble in water. In the QB compound the COOH of the fatty acid has been replaced by a basic nitrogen group, in the Igepons by a true sulfonic acid group. Nacconol, Aerosol, and Triton are likewise sodium salts of sulfonic acids. Their hydrophobic parts all contain ring structures. Triton is peculiar in that its hydrophobic part contains carbon atoms joined by ether linkages. Sodium glycholate is glycine conjugated to a sterol acid.

TABLE I

Amount of Detergent or Bile Salt Needed to Denature 10 Mg. Beef Methemoglobin in 10 Cc. of pH 6.8 Solution

Denaturant	Manufacturer	Amount of denaturant
		mg.
Duponol Special WA paste	Du Pont	2
Duponol PC	Du Pont	2
Igepon AP ex. conc.	General Dyestuffs	2
Arctic M salt free	Colgate	3
Nacconol NRSF	Monsanto	5
Igepon T powder	General Dyestuffs	6
QB	Du Pont	10
Aerosol OT	American Cyanamid	12
Duponol 80	Du Pont	20
Sodium glycholate	Merck	25
Sodium taurocholate (40 per cent)	Merck	25

As shown in Table I the exact amount of detergent needed to denature beef methemoglobin in neutral solution varies from one detergent to another. What is striking is that in all cases the amount required is

extremely small. All the other substances which have hitherto been shown to denature neutral isoelectric hemoglobin act only in much higher concentration. Thus 8 M urea denatures hemoglobin slowly whereas 0.0008 M Duponol PC denatures hemoglobin rapidly.

Of the substances hitherto shown to denature neutral hemoglobin salicylate is the most effective (Anson and Mirsky, 1934). Salicylate has a hydrophobic-hydrophilic structure but the hydrophobic part is much smaller than in substances with marked detergent properties. It would be interesting to test derivatives of salicylate in which the hydrophobic part has been made larger.

In the experiments summarized in Table I the concentration of hemoglobin is 1 mg. per cc. If more concentrated hemoglobin is used then more concentrated detergent is needed for denaturation. This means that when just enough detergent is added to bring about denaturation a large part of the detergent is combined with the protein.

The denaturation of methemoglobin in dilute solution by the various detergents is followed optically. Native methemoglobin is a brown compound with a band in the red. Denatured methemoglobin is a red compound with no band in the red.

The red color of denatured methemoglobin in Duponol PC solution does not change with time and is the same whether the Duponol PC is added to methemoglobin or to oxyhemoglobin. Thus the estimation of hemoglobin as denatured methemoglobin in Duponol PC is more satisfactory than the usual estimation of hemoglobin as acid hematin.

The denaturation of hemoglobin by Duponol PC can also be followed by solubility and digestibility tests. Denatured but not native hemoglobin is precipitated by 0.1 saturated ammonium sulfate and is digested by trypsin.

Previous Experiments with Detergents

Viruses.—Sreenivasaya and Pirie (1938) found that sodium dodecyl sulfate splits the large tobacco mosaic virus protein into smaller pieces and separates the protein part of the conjugated protein and the nucleic acid. The splitting is still incomplete several hours after a 1.0 per cent solution of crystalline sodium dodecyl sulfate is added to an equal volume of 1.0 per cent virus protein even at pH 8.0. At pH 7.0 the reaction is much slower still.

If Duponol PC is added to mosaic virus under the conditions described in Table I the virus is quite stable although methemoglobin under the same conditions is denatured in less than a minute. It may be that the viruses

in general are more resistant to synthetic detergents than ordinary proteins and that the detergents can therefore be used for the separation of virus proteins from ordinary proteins.

Sreenivasaya and Pirie observed further that tobacco mosaic protein split by sodium dodecyl sulfate and then dialyzed is precipitated by 0.1 saturated ammonium sulfate in which the native protein is soluble but is not digested by trypsin which digests denatured but not native virus protein. When the dialyzed protein is reprecipitated several times it becomes precipitable by ammonium sulfate in still lower concentration and becomes digestible by trypsin. Sreenivasaya and Pirie concluded from these observations that tobacco mosaic protein is not denatured by sodium dodecyl sulfate but that the protein part of the virus when separated from the nucleic acid part is less stable than the intact conjugated protein just as globin is less stable than hemoglobin and the prosthetic protein of the yellow enzyme is less stable than the yellow enzyme.

The evidence that tobacco mosaic virus is not denatured by sodium dodecyl sulfate cannot as yet be considered as conclusive.

Extraction of Visual Purple and Chlorophyll.—Bile salts have been used to extract the photosensitive pigment protein of the eye (Kühne, 1879) and to extract a chlorophyll compound from the chloroplasts of spinach (Smith, 1938). I have found that detergents such as Duponol PC extract chlorophyll much more effectively than the bile salts. It is now clear, however, that when bile salts and detergents are used to extract a protein, the protein extracted *may* not be in its original native undissociated form.

The visual purple extracted by bile salts has some of the properties of visual purple in the eye (Kühne, 1879) and it can be modified by denaturation procedures (Wald, 1935). This is evidence that visual purple is resistant to denaturation by bile salts. It is possible, however, that visual purple in the eye is a heavy weight protein similar to the tobacco mosaic virus. The effects of detergents and bile salts on visual purple are now being studied by Wald.

Because of their great penetrating and solvent powers the detergents will surely be used for the extraction of many biological substances. It will be important in each case to discover whether the substance extracted is modified by the detergent.

Physiological Effects of Bile Salts.—Bile salts emulsify fat, activate lipase, promote the absorption of various substances, and stimulate the flow of bile (Sobotka, 1937). All substances with the detergent type of hydrophobic-hydrophilic structure can emulsify fats. It may be that the bile

salts are simply biological detergent molecules whose specific structures are of secondary importance.

Lysis and Bactericidal Action.—The lysis of red blood cells and the killing and lysis of bacteria by detergents and bile salts have often been studied. Some of the widely used bactericidal agents, such as hexylresorcinol, have the detergent type of hydrophobic-hydrophilic structure.

Detergents of the Duponol series prevent the growth of Gram-positive bacteria even when the concentration of detergent is only 0.03 per cent (Cowles, 1938). Under the same conditions the growth of Gram-negative bacteria is not prevented.

I have found that if enough Duponol PC is added to red blood corpuscles not only are the cells lysed but the hemoglobin is denatured. It is likely that a detergent has some effects on both red cells and bacteria even when too little detergent is added to cause lysis and that if an excess of detergent is added to bacteria there is denaturation of enzyme proteins, some of which (White, 1938[2]), may be necessary for lysis. In studying the poisoning, killing, and lysis of bacteria by detergents it is desirable, therefore, that the detergent be added in a whole series of concentrations and not in only one concentration as is often done. The effects of detergents on bacteria are now being examined in detail by Dubos.

So far as I know there has been no systematic study of the toxicity and bactericidal powers of detergents *in vivo* or of the relative affinities of detergents for bacterial cells and for tissue cells and proteins. The *in vivo* experiments with pneumococcus summarized by White (1938[3]) were carried out before the modern detergents were available.

The relation between chemical structure and surface activity and bactericidal action is discussed by Bürgi and Laubenheimer (1931).

Secretion.—Substances with the detergent type of hydrophobic-hydrophilic structure promote secretion by the liver and the kidney (Höber, 1939).

It should be pointed out that there is no evidence at present that the effects of detergents on physiological systems are due to protein denaturation, although protein denaturation must now always be considered as a possibility.

Monolayers.—Effects of detergents on various monolayers have been described by Rideal (1939).

Detergent Micelles.—There are two interesting properties of the synthetic detergents themselves which should be called to the attention of biochemists.

[2] Chapter II (White, 1938).

[3] Chapter XIV (White, 1938).

As a result of three dimensional orientation the detergents form in solution large micelles of fairly definite size (Hartley, 1936). Each micelle consists of a hydrophobic core surrounded by a hydrophilic shell. There is an equilibrium between the large micelles and single detergent ions which, like the equilibrium between native and denatured protein, is extremely sensitive to temperature. In both the dissociation of the large detergent micelle and in the denaturation of protein many bonds of a single large molecule are broken in an all or none reaction. In the dissociation of the detergent micelle a large number of smaller molecules is formed and so the equilibrium is also sensitive to the detergent concentration.

Insoluble Potassium Salts of Detergents.—Some detergents, such as Duponol PC, form insoluble potassium salts under the same conditions under which both the sodium and calcium salts are soluble. Apparently the small difference between sodium and potassium can make a crucial difference in the formation of large insoluble complexes. This sort of phenomenon may account for the specific differences in the properties of sodium and potassium in biological systems.

Sreenivasaya and Pirie reported that phosphates and other buffers form insoluble compounds with sodium dodecyl sulfate. Presumably the potassium salts of the buffers were used.

EXPERIMENTAL

Preparation of Hemoglobin.—The preparation of hemoglobin for the estimation of proteinase (Anson, 1938) has now been slightly modified. Washed beef red blood corpuscles are shaken vigorously with a seventh their volume of toluene and allowed to stand a day or more at 5°C. The stromata and almost all the toluene are then removed by filtration with the aid of Hyflo Super-Cel (Johns-Manville). The filter cake is washed with a little water and the filtrate dialyzed in cellophane tubing as previously described. The remainder of the toluene is removed by this dialysis. If the toluene treatment is omitted a slime is formed on the cellophane which makes dialysis less efficient and there is sometimes bacterial growth.

Methemoglobin is prepared by the addition of an excess of ferricyanide and the excess ferricyanide is then removed by dialysis.

Optical Test for Denaturation.—The detergent or bile salt is added to 10 cc. of a solution containing 10 mg. of dialyzed beef methemoglobin and 1 drop of 1 M buffer containing equal parts Na_2HPO_4 and NaH_2PO_4. The detergent is neutralized if it is not neutral to begin with. Table I shows the amount of detergent or bile salt needed to cause the rapid disappearance of the band in the red. This procedure gives only rough comparative results which is all that was desired. The reaction can be followed quantitatively using a colorimeter and monochromatic green or red light. Green light is absorbed more strongly by denatured than by native methemoglobin (Anson and Mirsky, 1934). Red light is absorbed more strongly by native than by denatured methemoglobin. In

some cases, the denatured methemoglobin is partly dissociated into oxidized or ferri heme[4] and denatured globin.

Solubility and Digestibility Tests.—3 cc. of 10 per cent Duponol PC and 2 cc. of 1 M pH 6.8 phosphate buffer are added to 70 cc. of 2.5 per cent dialyzed beef oxyhemoglobin. The solution is dialyzed overnight against cold distilled water in a shaking dialyzer. The dialyzed denatured hemoglobin remains in solution, which shows the great affinity of Duponol PC for denatured hemoglobin,[5] and it has the same spectrum as before dialysis, with no band in the red. The dialyzed hemoglobin is completely precipitated by 0.1 saturated ammonium sulfate and is digested by trypsin about as readily as denatured hemoglobin in urea solution (Anson, 1938).

The excess Duponol PC is dialyzed away before the solubility and digestibility tests are carried out to avoid any interference by Duponol PC with the precipitation by salt and to avoid any inactivation of trypsin by Duponol PC.

Prevention of Trichloracetic Acid Precipitation.—0.2 N trichloracetic acid does not precipitate hemoglobin in 1 per cent solution if 1.3 per cent Duponol PC is present.

Colorimetric Estimation of Hemoglobin with Duponol PC.—The final solution contains 1 per cent Duponol PC, 0.04 M pH 6.8 sodium phosphate, and about 1 mg. of hemoglobin per cc. The colorimeter reading is the same 1 minute and 60 minutes after the Duponol is added. It is the same whether the Duponol is added to oxyhemoglobin or methemoglobin.

Lysis of Red Blood Cells.—0.2 cc. of centrifuged washed horse corpuscles is suspended in 5 cc. of 1 per cent NaCl solution. The addition of 1 drop of 10 per cent Duponol PC causes lysis. If too much Duponol PC is added not only are the corpuscles lysed but the hemoglobin is denatured.

Effects on Bacteria.—Bacterial growth does not appear in the 0.1 per cent hemoglobin solution containing 1 per cent Duponol PC, even if the solution is allowed to stand at 37°C. for weeks. In the absence of Duponol there is a rapid growth of bacteria under these conditions.

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[4] I should like to suggest the following terminology for the heme pigments:

$$\left.\begin{matrix}\text{hemo}\\\text{hemi}\end{matrix}\right\}\text{heme},\quad \left.\begin{matrix}\text{hemochromogen}\\\text{hemichromogen}\end{matrix}\right\}\text{hemechromogen},\quad \left.\begin{matrix}\text{hemoglobin}\\\text{hemiglobin}\end{matrix}\right\}\text{hemeglobin}.$$

[5] The combination between detergent and native protein has not yet been studied. Native tobacco mosaic virus is actually precipitated by Duponol PC.

The conditions for the dissociation of the compound between detergent and denatured protein have been found only very recently and so cannot be discussed here.

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THE REACTIONS OF DENATURED EGG ALBUMIN WITH FERRICYANIDE*

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(Received for publication, October 2, 1939)

INTRODUCTION

This paper describes the reactions between the reducing groups of denatured egg albumin and ferricyanide. Measurements have been made of the effects on the amount of ferricyanide reduced of varying the time, temperature, and pH of the reaction, and the concentration of ferricyanide; of adding the synthetic detergent, Duponol PC; and of treating the denatured protein before the ferricyanide reaction with formaldehyde and iodoacetamide, which are known to react with SH groups. The main result is that, provided the ferricyanide concentration is not too high and Duponol PC is present, there is a definite reaction between ferricyanide and denatured egg albumin. By a definite ferricyanide reaction is meant one in which the amount of ferricyanide reduced is, within wide limits, independent of the time, temperature, and pH of the reaction, and of the concentration of ferricyanide.

For the experiments on protein denaturation for which the definite reaction with ferricyanide was worked out and for many other practical applications of this reaction it is not necessary to know what protein groups reduce dilute ferricyanide in Duponol PC solution or how Duponol PC influences the reaction. The reactions between ferricyanide and various amino acids and proteins which will be described, however, indicate that the new definite reaction between ferricyanide and denatured egg albumin is a reaction with SH groups and that the effect of Duponol PC is to lower the ferricyanide concentration at which the SH groups of denatured egg albumin react with ferricyanide.

* A brief account of the reactions of denatured egg albumin with ferricyanide and of native egg albumin with iodine and iodoacetamide has been published in *Science* (Anson, 1939*a*).

HISTORICAL

The SH groups of denatured egg albumin have usually been studied by adding an oxidizing agent and measuring how much of the oxidizing agent is reduced. Thus it was found that 10 mg. of denatured egg albumin reduces about 0.0005 milliequivalents of cystine (Mirsky and Anson, 1935) and porphyrindin (Kuhn and Desnuelle, 1938; Greenstein, 1938); that is, the amounts of cystine and porphyrindin which would be reduced by 0.0005 mM of cysteine. Conditions were chosen under which all the SH groups and no other protein reducing groups were supposedly oxidized.

Mirsky and Anson also studied the SH groups of denatured egg albumin by an indirect method. Egg albumin was hydrolyzed with sulfuric acid and 0.0005 mM of cysteine was found in the hydrolysate of 10 mg. of albumin. If before the hydrolysis, the egg albumin was denatured, the denatured protein treated with ferricyanide or iodoacetate, and the excess ferricyanide or iodoacetate removed, then no cysteine was found on hydrolysis. Two conclusions were drawn from these results, that all the SH groups found on hydrolysis are free and reactive in denatured but unhydrolyzed egg albumin, and that all the free and reactive SH groups of denatured egg albumin are oxidized by cystine.

Greenstein found that denatured egg albumin in the presence of guanidine hydrochloride reduces about twice as much porphyrindin as heat denatured egg albumin in the absence of guanidine. The conclusion was drawn from this result that guanidine creates new SH groups in denatured egg albumin by breaking some as yet unidentified SH linkage.

Greenstein found that urea increases the amount of porphyrindin reduced by denatured egg albumin almost as much as guanidine. Although the results have never been published, I have used urea for many years to promote the reaction between protein reducing groups and the uric acid reagent.

Mirsky and Anson (1936) studied the reduction of ferricyanide by denatured egg albumin at pH 9.6. The amount of ferrocyanide formed was greater the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. Ferricyanide was reduced even if the SH groups of denatured egg albumin were first oxidized by cystine. At pH 9.6 ferricyanide was found to oxidize not only cysteine but also tyrosine and tryptophane. The amount of ferricyanide reduced by tyrosine and tryptophane was greater the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. It is known that in the oxidation of tyrosine a whole series of oxidation products can

be formed. From these results it was concluded that at pH 9.6 ferricyanide oxidizes tyrosine and tryptophane reducing groups of denatured egg albumin as well as SH groups.

The hydrolysate of 10 mg. of egg albumin contains 0.0015 mM of SH plus S—S sulfur. This value has been obtained by Kassell and Brand (1938) using their modification of Baernstein's method, and by S. Graff[1] using the Graff, Maculla, and Graff (1937) modification of the method of Vickery and White.

The Reactions between Denatured Egg Albumin and Ferricyanide

I have found that at pH 6.8 as at pH 9.6 the amount of ferricyanide reduced by denatured egg albumin is greater the higher the concentration

TABLE I

Reactions between Ferricyanide and 10 Mg. Denatured Egg Albumin

How denatured	Ferricyanide	Time	Temperature	pH	Ferrocyanide formed
	mM	*min.*	°*C.*		*mM*
HCl	0.002	10	37	6.8	0.00064
HCl	0.002	60	37	6.8	0.00075
HCl	0.05	10	37	6.8	0.00094
HCl	0.5	10	37	6.8	0.001
HCl	0.5	60	37	6.8	0.0012
HCl	0.002	10	37	9.6	0.00074
HCl	0.05	10	37	9.6	0.00123
Cl_3COOH	0.002	10	37	6.8	0.00063
Cl_3COOH	0.5	10	37	6.8	0.0011

of ferricyanide and the temperature and the longer the time of reaction (Table I). The difference between the results at pH 6.8 and at 9.6 is that a given amount of ferrocyanide is formed at a lower concentration of ferricyanide at pH 9.6 than at pH 6.8.

The synthetic detergent, Duponol PC (Du Pont), has a great effect on the reaction between denatured egg albumin and ferricyanide. Duponol PC denatures proteins such as hemoglobin and egg albumin and keeps the denatured protein in solution even at the isoelectric point. Whereas 8 M urea denatures neutral hemoglobin slowly, 0.0008 M Duponol PC denatures neutral hemoglobin rapidly (Anson, 1939*b*). Duponol PC is a more effective denaturant and solvent than most of the detergents I have tried and unlike many other detergents it does not reduce ferricyanide.

[1] Personal communication.

Since it is used in pharmaceutical products Duponol PC is the most carefully prepared of the Duponols.

Table II shows the amounts of ferrocyanide formed when ferricyanide is added under different conditions to egg albumin denatured by Duponol PC.

TABLE II

Reactions between Ferricyanide and 10 Mg. Denatured Egg Albumin in Duponol PC Solution

Ferricyanide	Time	Temperature	pH	Duponol PC	Ferrocyanide formed
mM	*min.*	°*C.*		*mg.*	*mM*
0.001	2	37	6.8	50	0.00082
0.001	10	37	6.8	50	0.00096
0.001	60	37	6.8	50	0.00098
0.001	10	37	9.6	50	0.00096
0.002	2	37	6.3	10	0.00093
0.002	10	37	6.3	10	0.00099
0.002	2	0	6.8	10	0.00055
0.002	10	0	6.8	10	0.00080
0.002	100	0	6.8	10	0.00095
0.002	2	37	6.8	10	0.00092
0.002	10	37	6.8	10	0.00096
0.002	100	37	6.8	10	0.00098
0.002	2	37	6.8	50	0.00097
0.002	10	37	6.8	50	0.00098
0.002	100	37	6.8	50	0.00105
0.002	2	37	9.6	50	0.00095
0.002	10	37	9.6	50	0.00099
0.002	2	100	6.8	50	0.00095
0.002	10	100	6.8	50	0.00100
0.05	2	37	6.8	10	0.00094
0.05	10	37	6.8	10	0.00096
0.05	10	37	6.8	50	0.00098
0.05	10	37	9.6	10	0.00125
0.2	60	37	6.8	10	0.00105
0.2	20	60	6.8	10	0.00124
0.2	2	100	6.8	10	0.00110
0.2	10	100	6.8	10	0.00136
0.2	20	100	6.8	10	0.00150

First, 0.001 mM of ferrocyanide is formed from 0.001 mM of ferricyanide whereas in the absence of Duponol PC only 0.00064 mM of ferrocyanide is formed at pH 6.8 from 10 mg. of denatured egg albumin and 0.002 mM of ferricyanide (Table I). Thus Duponol PC increases the amount of ferrocyanide formed from ferricyanide in dilute solution.

Secondly, if the ferricyanide concentration is not too high, then 0.001

mM of ferrocyanide is formed despite wide variations in the concentrations of ferricyanide and Duponol PC, the temperature, the time of reaction, and the pH. Thus in the presence but not in the absence of Duponol PC there is a definite reaction between denatured egg albumin and dilute ferricyanide.

Finally, if enough ferricyanide is added, then more than 0.001 mM of ferrocyanide is formed and the amount of ferrocyanide formed is greater, the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. Thus in the presence as well as in the absence of Duponol PC there is an indefinite reaction between denatured egg albumin and ferricyanide, but in the case of egg albumin Duponol PC makes possible a separation between the definite reaction with dilute ferricyanide and the indefinite reaction with concentrated ferricyanide.

At pH 9.6 the results are qualitatively the same as at pH 6.8. At both pH's there is a definite reaction with dilute ferricyanide and an indefinite reaction with more concentrated ferricyanide. At pH 9.6, however, the indefinite reaction begins at a lower ferricyanide concentration than at pH 6.8.

Formaldehyde and iodoacetamide are known to react with SH groups. If denatured egg albumin is first treated with formaldehyde or iodoacetamide, and dilute ferricyanide is then added in neutral Duponol solution, no ferrocyanide is formed.

Reactions of Ferricyanide with Amino Acids and Cysteine-Free Proteins

Before discussing the question of what protein groups react with ferricyanide I shall summarize what is known from previous experiments and from new experiments about the reactions of ferricyanide under various conditions with amino acids and with proteins which do not contain cysteine. The results of the new experiments are given in Table III.

Ferricyanide in neutral solution oxidizes to S—S the SH of glutathione. One molecule of ferrocyanide is formed for each SH group which is oxidized. The ferrocyanide formed can be estimated as Prussian blue (Mason, 1930).

Similarly ferricyanide oxidizes cysteine to cystine. As shown in Table III this oxidation of cysteine takes place even at pH 3.2. Mason found the oxidation of glutathione at pH 3.2 to be slow and incomplete. Thus the ease with which SH groups are oxidized depends on the structure of the whole molecule. Similarly iodoacetate reacts more readily with some SH compounds than with others (Michaelis and Schubert, 1934; Smythe, 1936).

In neutral solution containing Duponol PC, ferricyanide, in dilute solu-

tion, does not react with cystine, tyrosine, tryptophane, or with proteins such as serum albumin, pepsin, and chymotrypsinogen which do not con-

TABLE III

Reactions between Ferricyanide and Amino Acids and Cysteine-Free Proteins

Amino acid or protein	Ferricyanide	Time	Temperature	pH	Duponol PC	Ferrocyanide formed
	mM	*min.*	°*C.*		*mg.*	*mM*
0.001 mM cysteine	0.002	10	37	1.0	0	0.00018
0.001 mM cysteine	0.002	10	37	1.0	50	0.00016
0.001 mM cysteine	0.002	10	37	2.0	0	0.00023
0.001 mM cysteine	0.002	10	37	2.0	50	0.00025
0.001 mM cysteine	0.002	10	37	3.0	0	0.00098
0.001 mM cysteine	0.002	10	37	3.0	50	0.00092
0.001 mM cysteine	0.002	10	37	6.8	0	0.00098
0.001 mM cysteine	0.002	10	37	9.6	0	0.00101
0.001 mM cysteine	0.002	10	37	9.6	50	0.00101
0.001 mM cysteine	0.05	10	37	9.6	50	0.00104
0.001 mM cysteine	0.2	20	50	6.8	10	0.00104
0.001 mM cysteine	0.2	20	80	6.8	10	0.00149
0.001 mM cysteine	0.2	10	37	9.6	50	0.00144
0.0005 mM cystine	0.01	10	37	9.6	50	0.0
0.0005 mM cystine	0.1	10	37	9.6	50	<0.00005
0.0005 mM cystine	0.2	20	50	6.8	10	0.0001
0.0005 mM cystine	0.2	20	80	6.8	10	0.00062
0.0005 mM cystine	0.2	20	100	6.8	10	0.00144
0.0005 mM cystine	0.5	20	80	6.8	10	0.00093
1 mg. tyrosine	0.002	10	37	6.8	25	0.0
1 mg. tyrosine	0.02	10	100	6.8	25	<0.0001
1 mg. tyrosine	0.1	10	100	6.8	50	0.0009
1 mg. tyrosine	0.2	10	37	6.8	50	<0.0001
0.001 mM tyrosine	0.002	10	37	9.6	50	<0.00005
0.001 mM tyrosine	0.01	10	37	9.6	50	0.00116
0.001 mM tyrosine	0.1	10	37	9.6	50	0.00175
0.001 mM tryptophane	0.01	10	37	6.8	0	0.0
0.001 mM tryptophane	0.1	10	37	6.8	0	<0.00005
0.001 mM tryptophane	0.1	10	70	6.8	0	<0.00005
0.001 mM tryptophane	0.5	10	37	6.8	0	0.0002
20 mg. serum albumin	0.003	10	37	6.8	50	0.0
20 mg. serum albumin	0.2	10	100	6.8	50	0.004
10 mg. pepsin	0.003	10	37	6.8	50	0.0
10 mg. chymotrypsinogen	0.002	10	37	6.8	50	0.0
10 mg. chymotrypsinogen	0.2	20	37	6.8	50	<0.00005
10 mg. chymotrypsinogen	0.2	20	50	6.8	10	0.00038
10 mg. chymotrypsinogen	0.2	20	100	6.8	10	0.00168

tain cysteine. At pH 9.6 the reactions with dilute ferricyanide are very slight.

Concentrated ferricyanide, however, oxidizes cystine, tyrosine, tryptophane, serum albumin, and chymotrypsinogen. The amount of ferrocyanide formed is greater the higher the temperature and the concentration of ferricyanide. At pH 9.6 the reactions begin at a lower ferricyanide concentration than at pH 6.8.

Thus cysteine is the only amino acid which is known to give a definite stoichiometric reaction with ferricyanide and which is known to react with dilute neutral ferricyanide at all. In the few cases which have been studied, proteins which do not contain cysteine do not react with dilute ferricyanide in neutral Duponol PC solution. If the concentration of ferricyanide is high enough there is an indefinite reaction of ferricyanide with cystine, tyrosine, tryptophane, and with proteins which contain these other amino acids but not cysteine. The indefinite reaction begins at a lower ferricyanide concentration at pH 9.6 than at pH 6.8.

The reaction, if any, of the carbohydrate component of egg albumin (Neuberger, 1938) with ferricyanide in Duponol solution has not been studied.

DISCUSSION

The evidence that the definite reaction between dilute ferricyanide and denatured egg albumin in neutral Duponol PC solution is due solely to the oxidation of SH to S—S is as follows:

1. Dilute ferricyanide in neutral solution oxidizes cysteine to cystine. This reaction, like the definite reaction between dilute ferricyanide and denatured egg albumin in Duponol PC solution, is within wide limits independent of the concentration of ferricyanide and the pH.

2. Cystine, tyrosine, and tryptophane and proteins containing these amino acids, but not cysteine, do not react with dilute ferricyanide in Duponol PC solution.

3. Under those conditions under which ferricyanide does react with cystine, tyrosine, tryptophane, and cysteine-free proteins, the reaction is not a definite reaction.

4. Formaldehyde and iodoacetamide abolish the reaction of denatured egg albumin with dilute ferricyanide. These two reagents are not known to react with any reducing groups other than SH groups.

Very little is known about the effects of protein structure on the properties of amino acid groups. It is possible that there are some peculiarly reactive cystine, tyrosine, or tryptophane groups in denatured egg albumin. Very little is known about the oxidation of amino acid reducing groups in complex systems which contain different types of reducing groups.

It is possible that there are in denatured egg albumin cystine, tyrosine, or tryptophane groups which are oxidized by dilute ferricyanide in the presence but not in the absence of SH groups.

The sulfuric acid hydrolysate of 10 mg. of egg albumin was found to contain 0.0005 mM of cysteine (Mirsky and Anson, 1935). Some cysteine was probably lost by oxidation, decomposition, and adsorption by humin. If future experiments, however, show that 10 mg. of egg albumin contains only 0.0005 mM of cysteine then one of three assumptions must be made to explain the further fact that 10 mg. of egg albumin reduces 0.001 mM of dilute ferricyanide in Duponol PC solution. The reduction of ferricyanide may not be due solely to SH groups; new SH groups may be produced by Duponol PC which do not exist in native egg albumin and are not liberated by acid hydrolysis; or there may be an equilibrium between free SH groups and linked SH groups and when the free SH groups are oxidized more free SH groups are formed to maintain the equilibrium.

Thus, all the present experiments are in harmony with the simple conclusion that the oxidation of denatured egg albumin in Duponol PC solution by dilute ferricyanide is due to the oxidation of SH to S—S groups. Other and more complex mechanisms for the reduction of ferricyanide are, however, conceivable. All these conceivable mechanisms cannot be definitely excluded so long as only the formation of ferrocyanide is measured.

The evidence that there are in denatured egg albumin free SH groups which react with concentrated ferricyanide and iodoacetamide in the absence of Duponol PC but not with dilute ferricyanide and that therefore the effect of Duponol PC is merely to lower the concentration of ferricyanide at which ferricyanide reacts with the SH groups of denatured egg albumin is as follows:

1. The amount of ferricyanide reduced by denatured egg albumin in the absence of Duponol PC solution is greater the higher the concentration of ferricyanide, even when the ferricyanide is so dilute that it does not react with cystine, tyrosine, or tryptophane.

2. Denatured egg albumin treated with iodoacetamide in the absence of Duponol PC no longer reduces dilute ferricyanide in the presence of Duponol PC.

The evidence that the indefinite reaction between concentrated ferricyanide and denatured egg albumin in Duponol PC solution is a reaction with cystine, tyrosine, or tryptophane groups is as follows:

1. In the indefinite reaction between concentrated ferricyanide and denatured egg albumin as in the indefinite reaction between concentrated ferricyanide and cystine, tyrosine, tryptophane, and cysteine-free pro-

teins, more ferrocyanide is formed the higher the concentration of ferricyanide and the temperature and the more alkaline the solution.

2. The indefinite reaction between concentrated ferricyanide and denatured egg albumin begins under roughly the same conditions as the indefinite reaction between concentrated ferricyanide and denatured serum albumin and chymotrypsinogen. In both cases, the indefinite reaction begins at a lower ferricyanide concentration at pH 9.6 than at pH 6.8.

Altogether the facts about the reactions of ferricyanide and denatured egg albumin are now in clear and useful form and they have been described quite separately from the conclusions which provide at present the simplest explanation of the facts. It cannot be said that the conclusions have been definitely proven. There is no reagent available which one can be sure reacts with all the SH groups of denatured egg albumin and with no other groups. More certain conclusions will be possible only when some specific reagent is discovered or when the reducing groups of many different proteins are studied more extensively by a number of entirely independent methods.

Greenstein's conclusion that guanidine creates new SH groups by breaking some SH linkage was based on the observation that guanidine increases the porphyrindin titration and on the assumption that even in the absence of guanidine, all the free SH groups of denatured egg albumin are oxidized in the porphyrindin titration. No experimental evidence was given in support of this assumption. The titrations were carried out under only one arbitrarily chosen set of conditions. The concentration of porphyrindin, the time, temperature, and pH of the reaction were not varied. Indeed the instability of porphyrindin sets definite limits to the conditions under which porphyrindin can be used. Furthermore, it is difficult to estimate small amounts of reduced porphyrindin in the presence of large amounts of oxidized porphyrindin.

The ferricyanide experiments suggest by analogy that there are free SH groups in denatured egg albumin which do not react rapidly with the dilute porphyrindin used by Greenstein but which would react with more concentrated porphyrindin and that the effect of guanidine is not to create new SH groups by the breaking of hypothetical SH linkages but to lower the concentration of porphyrindin at which porphyrindin reacts rapidly with the SH groups of denatured egg albumin.

It should be emphasized that there is no contradiction between the porphyrindin experiments themselves and the more varied experiments with ferricyanide at different concentrations and with iodoacetamide. The difference between Greenstein's conclusion and the conclusion sug-

gested by the experiments with ferricyanide and iodoacetamide is a difference in the interpretation of how guanidine and Duponol PC influence the reducing power of denatured egg albumin.

EXPERIMENTAL

Reagents

Egg albumin is crystallized with ammonium sulfate, recrystallized three times, dialyzed against cold distilled water in a shaking dialyzer, and stored frozen. The nitrogen content of the albumin solution is estimated by the Kjeldahl method.

Acid ferric sulfate containing phosphoric acid and gum ghatti is prepared according to Folin and Malmros (1929).

Potassium ferricyanide as obtained commercially always contains some ferrocyanide and some commercial preparations contain a blue impurity. The ferrocyanide in a 0.5 M solution of Merck's reagent potassium ferricyanide is oxidized with just not enough bromine water to oxidize all the ferrocyanide. It is possible to obtain a preparation of ferricyanide treated with bromine such that the addition of 0.5 cc. ferric sulfate solution to 5 cc. of 0.5 M ferricyanide treated with bromine gives an increase in light absorption due to the formation of Prussian blue which is detectable with the photoelectric colorimeter but which is 5 times less than the increase obtained when 1 drop 0.001 M ferrocyanide is added before the ferric sulfate. The ferricyanide is stored at 5°C. in the dark and its ferrocyanide content occasionally checked.

Iodoacetamide.—50 gm. of chloracetamide (Eastman) and 80 gm. of NaI are dissolved in 1 liter of acetone with gentle warming. The solution is allowed to stand 2 days at room temperature, the precipitate of NaCl is filtered off, and the acetone is boiled off *in vacuo*. When the temperature begins to rise rapidly, the evaporation is stopped, and the solution is cooled in ice water. The crystals which are formed are filtered off in the cold and washed with ice cold acetone. The same process of evaporation, etc. is repeated twice more with the filtrates and the washings. Thus decomposition of the iodoacetamide by hot acetone is avoided and an acetone washed product is obtained without too much loss.

The crystals are dried with a current of air and added to an equal weight of water which is warmed rapidly until the crystals are dissolved. The solution is then promptly cooled with ice water and the crystals filtered off in the cold. The crystals are dried in a desiccator and the recrystallization repeated.

Iodoacetamide prepared according to the above directions gives no iodine test with starch and no precipitate with acid silver nitrate, which indicates the absence of iodide and of iodoacetic acid which slowly liberates iodide in acid solution. Iodoacetamide is slowly converted into iodoacetic acid in neutral and alkaline solution.

Duponol.—The Duponols (Du Pont) have the general composition $CH_3(CH_2)_n$-CH_2OSO_3Na. Duponol Special WA Paste consists mainly of the C_{12} compound which is called sodium dodecyl sulfate. The dried form of Special WA Paste is called Duponol ME Dry. The "sodium dodecyl sulfate" used in the present experiments was a dried product kindly provided by Proctor and Gamble. It is not available commercially. Duponol WA Paste is a mixture of the C_{10}-C_{18} compounds and contains small amounts of the free alcohols and Na_2SO_4. Duponol PC is a dried and purified form of Duponol WA Paste from which the free alcohols have been extracted with acetone.

Duponol PC and sodium dodecyl sulfate can be kept as 10 per cent stock solutions.

Buffers.—Sodium buffer salts are used rather than potassium salts which precipitate Duponol. Potassium ferricyanide is used rather than sodium ferricyanide only because satisfactory sodium ferricyanide is not available commercially. Potassium ferricyanide precipitates Duponol PC if more than 0.2 mM is used in the experiments to be described.

The 1.0 M pH 6.8 phosphate buffer is made up from equal parts 1 M Na_2HPO_4 and NaH_2PO_4. The pH 6.3 buffer is made up of 1 part 1 M Na_2HPO_4 and 3 parts 1 M NaH_2PO_4.

The pH 9.6 borate buffer is made up from 7 parts 1 M sodium borate and 3 parts 1 M NaOH.

The Reactions between Ferricyanide and Denatured Egg Albumin

Table I gives the amounts of ferrocyanide formed when ferricyanide is added under various conditions to egg albumin denatured by hydrochloric or trichloracetic acid.

I shall first describe the experiments with egg albumin which has been denatured by HCl and then brought to pH 6.8.

1 cc. of 1 N HCl is added to 4 cc. of 3 per cent dialyzed egg albumin. The test tube containing the solution is placed in 50°C. water for 2 minutes and then is cooled. The solution is neutralized with NaOH to green to brom thymol blue and then diluted to 12 cc.

To 1 cc. of the diluted solution containing 10 mg. of denatured egg albumin are added 0.1 cc. of pH 6.8 phosphate buffer and 1 cc. of ferricyanide solution. After the ferricyanide reaction, the solution is diluted to 9 cc. and mixed with 1 cc. of 2 N trichloracetic acid. The precipitate is filtered off. To 5 cc. of the filtrate is added 0.5 cc. of water, or if the amount of ferricyanide present is less than 0.05 mM 0.5 cc. of a ferricyanide solution containing enough ferricyanide to make the final amount 0.05 mM. Finally Prussian blue is developed by the addition of 0.3 cc. of the ferric sulfate solution and the color is read after 20 minutes against a ferrocyanide standard or against a blue glass calibrated with a ferrocyanide standard. A red color filter is used because red light is strongly absorbed by Prussian blue and weakly absorbed by ferricyanide.

The ferrocyanide standard is made up by adding ferrocyanide to neutralized egg albumin, adding trichloracetic acid, and adding before filtration the same amount of ferricyanide used in the experiment. Ferric sulfate is added to the filtrate as already described.

Although ferricyanide increases the rate at which Prussian blue is formed (why, I do not know) it does not affect significantly the amount of red light absorbed until the amount of ferricyanide is 0.2 mM or more. The amount of Prussian blue formed is the same whether the albumin, trichloracetic acid, and filtration are omitted or not, provided there is no impurity of ferric salts in the reagents. Any Prussian blue which is formed from ferric salts before filtration is removed by filtration.

The experiments at pH 9.6 are carried out exactly as the experiments at pH 6.8 with two exceptions. Before the acid denatured albumin is diluted to 12 cc. it is made pink to phenolphthalein instead of green to brom thymol blue. And 0.3 cc. of borate buffer is added instead of 0.1 cc. of phosphate buffer.

When the egg albumin is denatured by trichloracetic acid instead of by hydrochloric acid 1 cc. of 2 N trichloracetic acid is added instead of 1 cc. of 1 N hydrochloric acid. The suspension is allowed to stand 5 minutes at room temperature and is then neutralized. Otherwise the procedure is exactly that already described.

Table II gives the amounts of ferrocyanide formed when ferricyanide is added under various conditions to denatured egg albumin in the presence of Duponol PC. A few experiments carried out with sodium dodecyl sulfate gave exactly the same results as the experiments with Duponol PC.

I shall first describe the experiments at pH 6.8 and pH 6.3. 0.5 cc. of Duponol PC solution is added to 1 cc. of 1 per cent dialyzed egg albumin. After 5 minutes, 0.2 cc. of phosphate buffer and 1 cc. of ferricyanide are added. After the ferricyanide reaction there are added 0.5 cc. 2 N H_2SO_4, enough Duponol PC solution to make the final amount of Duponol 50 mg., enough ferricyanide to make the final amount 0.05 mM, water to 9.5 cc., and finally 0.5 cc. of ferric sulfate to develop the Prussian blue. Precipitation of the protein by ferric sulfate is prevented by the 50 mg. of Duponol PC.

The ferrocyanide standard is made up in the presence of the amounts of albumin, Duponol PC, sulfuric acid, and ferricyanide used in the experiment. Albumin, Duponol, and ferricyanide in amounts less than 0.2 mM have little effect on the Prussian blue color. The ferricyanide is added after the solution has been acidified with sulfuric acid.

When 0.2 mM or more of ferricyanide is present and the temperature is 80°C. or higher, a small amount of ferrocyanide is formed in the absence of albumin. The ferrocyanide standard is therefore made up by adding ferrocyanide to a ferricyanide-Duponol PC solution which has been heated in exactly the same way as the ferricyanide-Duponol-albumin solution.

The experiments at pH 9.6 are carried out as follows. There are added to 1 cc. of 1 per cent dialyzed egg albumin 1 cc. of ferricyanide solution, 1 drop of 0.5 N NaOH, and 0.3 cc. of borate buffer. This solution is warmed to 37°C. and there is added to it 0.5 cc. of Duponol PC solution previously warmed to 37°C. Thus the denatured egg albumin is not exposed to air before it is exposed to ferricyanide. The experiment is completed in exactly the same manner as the experiments in neutral solution.

Formaldehyde.—To 1 cc. of 1 per cent dialyzed egg albumin there are added 0.5 cc. of 2 per cent Duponol PC, 2 drops of 0.5 N NaOH, and 6 drops of 37 per cent formaldehyde. After the solution has stood 5 minutes at room temperature there are added 0.2 cc. pH 6.8 phosphate, 1 drop of 1 N HCl, and 1 cc. of 0.05 M ferricyanide. The solution is allowed to stand 10 minutes at 37°C. and the ferric sulfate test is carried out as previously described. No Prussian blue is obtained.

Formaldehyde alone does not interfere with the Prussian blue reaction and does not reduce dilute ferricyanide at 37°C. Formaldehyde does reduce concentrated ferricyanide at 100°C.

Iodoacetamide.—Iodoacetamide is added to a neutral solution of egg albumin denatured by HCl or Duponol PC and the reducing power of the denatured egg albumin which has been treated with iodoacetamide is then tested with ferricyanide.

1 cc. of 1 N HCl is added to 4 cc. of 3 per cent dialyzed egg albumin. The test tube containing the solution is placed in 50°C. water for 2 minutes. The solution is cooled, neutralized with NaOH to green to brom thymol blue, and there are added 0.6 cc. pH 6.8 phosphate buffer, 2 cc. of 0.06 M iodoacetamide, and water to 12 cc. The solution is allowed to stand various times at 37°C. before samples are taken for the ferricyanide test. For the ferricyanide test there are added to 1 cc. of the albumin-iodoacetamide solution 3 drops of 1 M NaH_2PO_4, 0.3 cc. of 1 M pH 6.3 phosphate buffer, 0.5 cc. of 0.2 M ferricyanide, and 0.5 cc. of 10 per cent Duponol PC. After the solu-

tion has stood 10 minutes at 37°C. the ferric sulfate is added for the development of Prussian blue.

If the denatured albumin is treated for 5 minutes with iodoacetamide, about 0.0002 mM of ferrocyanide is formed. If the iodoacetamide reaction is carried out for 30 minutes, no ferrocyanide is formed.

As a control, the acid denatured egg albumin is allowed to stand 30 minutes in neutral solution in the absence of iodoacetamide. Then Duponol and ferricyanide are added and the normal 0.001 mM of ferrocyanide obtained. The control experiment shows that the reducing groups of denatured egg albumin are not appreciably oxidized by air in 30 minutes.

The experiment with egg albumin denatured by Duponol PC is carried out as follows. To 1 cc. of 4 per cent dialyzed egg albumin there are added 1 drop of 0.5 N NaOH, 0.3 cc. of pH 6.8 phosphate buffer, 1 cc. of 10 per cent Duponol PC, and 0.6 cc. of 0.06 M iodoacetamide and water to 4 cc. 1 cc. samples are removed after the solution has been allowed to stand for various times at 37°C. To the 1 cc. sample there are added 1 drop of NaH_2PO_4, 0.3 cc. of pH 6.3 phosphate, 0.5 cc. of 0.2 M ferricyanide, and 0.5 cc. of 5 per cent Duponol PC. The ferric sulfate test is carried out after the solution has stood 10 minutes at 37°C.

If the denatured egg albumin is treated with iodoacetamide for 30 minutes in neutral Duponol PC, about 0.0001 mM of ferrocyanide is still formed from the ferricyanide. If the iodoacetamide reaction is carried out for 180 minutes no Prussian blue is obtained. Whether Duponol PC has a small direct effect on the iodoacetamide reaction which slows it up, or changes the pH slightly, has not been investigated.

As a control, the denatured egg albumin is allowed to stand 180 minutes in neutral Duponol solution in the absence of iodoacetamide and then a mixed solution of ferricyanide and iodoacetamide is added either at pH 6.8 or at pH 6.3. Again 0.001 mM of ferrocyanide is obtained, as would be obtained in the absence of iodoacetamide. This control shows not only the stability of the SH group in the absence of iodoacetamide but also shows that iodoacetamide does not interfere with the ferricyanide reaction. At pH 6.3, 0.00095 mM ferrocyanide is formed even when the ferricyanide is added 1 minute after the iodoacetamide.

Table III shows the amounts of ferrocyanide formed when ferricyanide is added under various conditions to several amino acids and to several proteins which do not contain cysteine. The experiments are carried out in exactly the same way as the experiments with egg albumin.

SUMMARY

The following facts have been established experimentally.

1. In the presence of the synthetic detergent, Duponol PC, there is a definite reaction between *dilute* ferricyanide and denatured egg albumin. 0.001 mM of ferrocyanide is formed by the oxidation of 10 mg. of denatured egg albumin despite considerable variation in the time, temperature, and pH of the reaction and in the concentration of ferricyanide.

2. If the concentration of ferricyanide is sufficiently high, then the

reaction between ferricyanide and denatured egg albumin in Duponol solution is indefinite. More ferrocyanide is formed the longer the time of reaction, the higher the temperature, the more alkaline the solution, and the higher the concentration of ferricyanide.

3. Denatured egg albumin which has been treated with formaldehyde or iodoacetamide, both of which abolish the SH groups of cysteine, does not reduce dilute ferricyanide in Duponol PC solution.

4. Cysteine is the only amino acid which is known to have a definite reaction with ferricyanide or which is known to react with dilute ferricyanide at all. The cysteine-free proteins which have been tried do not reduce dilute ferricyanide in Duponol PC solution.

5. Concentrated ferricyanide oxidizes cystine, tyrosine, and tryptophane and proteins which contain these amino acids but not cysteine. The reactions are indefinite, more ferrocyanide being formed, the higher the temperature and the concentration of ferricyanide.

6. The amount of ferrocyanide formed from denatured egg albumin and a given amount of ferricyanide is less in the absence than in the presence of Duponol PC.

7. The amount of ferrocyanide formed when denatured egg albumin reacts with ferricyanide in the absence of Duponol PC depends on the temperature and ferricyanide concentration throughout the whole range of ferricyanide concentrations, even in the low range of ferricyanide concentrations in which ferricyanide does not react with amino acids other than cysteine.

The foregoing results have led to the following conclusions which, however, have not been definitely proven.

1. The definite reaction between denatured egg albumin in Duponol PC solution and dilute ferricyanide is a reaction with SH groups whereas the indefinite reactions with concentrated ferricyanide involve other groups.

2. The SH groups of denatured egg albumin in the absence of Duponol PC react with iodoacetamide and concentrated ferricyanide but they do not all react rapidly with dilute ferricyanide.

3. Duponol PC lowers the ferricyanide concentration at which the SH groups of denatured egg albumin react with ferricyanide. The SH groups of denatured egg albumin, however, are free and accessible even in the absence of Duponol PC.

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ON THE PERMEABILITY OF THE STOMACH MUCOSA FOR ACIDS AND SOME OTHER SUBSTANCES

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INTRODUCTION

The main object of these studies has been the collecting of further experimental evidence for the view that the living stomach mucosa shows in many respects the behavior of a dialysis or diffusion membrane (Teorell, 1933).

In a previous work the author (Teorell, 1933) had conclusively proved that the acidity of HCl, introduced into the cat's stomach, was reduced by a process which appeared to be ordinary diffusion.

Some other electrolytes were also found to diffuse through the mucosa surface. Not only could many ions penetrate into the mucosa from the stomach content, but there was also a simultaneous, outwardly directed diffusion (in the main consisting of alkali chlorides) from the mucosa cells (or from the blood across the mucosa) into the stomach content.

No attempt will be made here to mention all the papers concerned with this field, as the subject has recently been well reviewed by Hollander and by Ihre. The older literature before 1933 was sketched by Gelman and Scheweluchin and by the author (1933). Papers with a direct bearing on the present communication will be referred to in the discussions below.

EXPERIMENTAL

Adult cats were fasted 24 hours and narcotized first with ether, then with chloralose-urethan given intravenously. Ligatures were tied round the pylorus and the cardia. A cannula was tied in the distal pyloric part of the stomach. A thin rubber tubing with many holes was introduced through the cannula when juice samples were to be taken, with a syringe serving as aspirator. The stomach was flushed inside with many portions of warm saline, emptied and allowed to come to rest during 1 to 2 hours before the actual experiments began. As a rule no secretion was found after that period.

* The experiments were carried out when the author held a fellowship of The Rockefeller Foundation.

The solutions to be examined were measured accurately (to ±0.1 cc.), introduced into the emptied stomach cavity, and allowed to stay there for 15 minutes when the fluid was aspirated completely and again measured. After a small amount (generally 0.1 to 0.3 cc.) had been withdrawn for quantitative analysis, the rest of the fluid was poured back. After a further interval of 15 minutes the whole procedure was repeated. HCl secretion was induced by subcutaneous injection of histamine. A histamine secretion test was always used as a control of the intactness of the stomach after the conclusion of long experiments.

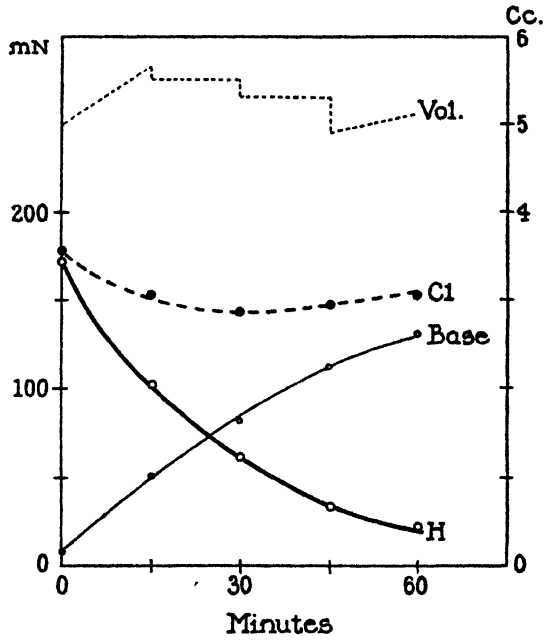

FIG. 1. Introduction of isotonic hydrochloric acid in a cat's stomach. Note the "exponential" decrease of the acidity (H) and the simultaneous increase of alkali cations (base) without appreciable volume change, indicating an exchange diffusion process (*cf.* Fig. 8). The temporary chloride decrease is also in accordance with the diffusion concept (*cf.* Teorell (1933, p. 291)).

The acidity was determined by microtitration using brom-thymol blue (pH 7.0) as indicator. Total chlorides were electrometrically titrated in the same sample using $AgNO_3$. Acetate was determined using micro-conductometrical titration in 50 per cent alcohol employing mercuric perchlorate.[1] Sulfate analysis was also performed conductometrically using Ba-acetate.[1] Phosphate was determined spectrophotometrically (Teorell, 1931), iodate iodimetrically, glycine after wet combustion as NH_3 by Nesslerization, and glucose with the aid of Benedict's method.

All concentrations are expressed in millinormality (mN).

[1] Britton, H. T., Conductometric analysis, London, Chapman and Hall, 1934, 115 (acetate), 109 (sulfate).

RESULTS

In order to simplify the presentation of the experimental results they are all plotted graphically.

Hydrochloric Acid, HCl, (Fig. 1). The acidity (H ions) decreased rapidly following an "exponential" curve. The Cl concentration also fell off somewhat during the first stages, but after about half an hour the Cl level showed a tendency to recover to its initial value of 160–170 mN. The "*base*," consisting mainly of Na, rose at a rate which was slower than the disappearance of the H ions.

During these changes of the ionic concentrations, the volume of the introduced samples underwent but slight variation. With the exception of the first 15 minute interval when a 15–20 per cent increase took place, the volume remained practically constant.

These observations were confirmed in numerous duplicate experiments on other cats: in Fig. 2 there are collected six similar experiments with 5 cc. samples and one experiment with a 10 cc. sample. In the 5 cc. curve the H and Cl run, on the whole, closely parallel. In the 10 cc. curve the H decreased at a rate about half that of the 5 cc. curve. Further experiments with still larger volumes showed very little decrease of acidity during the time of the experiment.

Perchloric Acid, $HClO_4$, (Fig. 3). The acidity decreased rapidly at a rate approximately equal to that of HCl without any significant increase or decrease of the introduced sample volumes. At the same time a continuous increase in Cl ions was observed.

Sulfuric Acid, H_2SO_4, (Fig. 3). Here a complete analysis of all ionic constituents was possible. The H decreased somewhat more slowly than with HCl or $HClO_4$. The SO_4 ions decreased in concentration although not rapidly. Cl and base increased steadily. It is noticeable that the total base (Cl + SO_4) remained practically constant at a value about 170–180 mN (blood isotonicity?). Irregular, but comparatively small volume changes were observed.

A comparison between a strong and some weak acids (HCl, lactic acid, formic acid, and acetic acid), (Fig. 4). Equal volumes (in this case 10, not 5 cc.) of the different acids were compared on the same animal. It is striking that the titratable acidity of the weaker acids diminished far more quickly than for HCl. Simultaneously the Cl increase was more marked when testing the weak acids. The volume changes were small.

Acetic Acid and Sodium Acetate, (Fig. 5). Pure HAc, an equimolecular mixture of HAc and NaAc, and a pure NaAc solution were tested. The

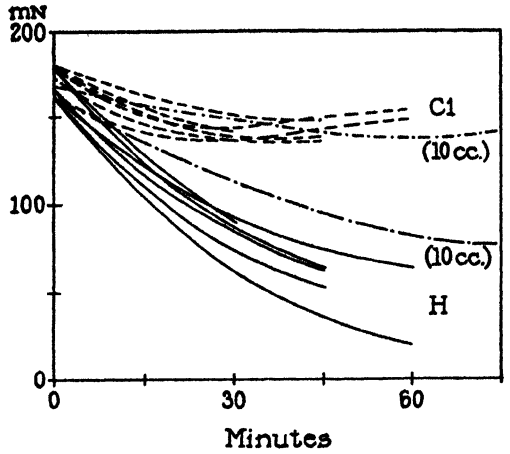

FIG. 2. Results of HCl introduction in six cats. In all cases but one 5 cc. were used. Observe that the acidity drop is slower in the 10 cc. experiment.

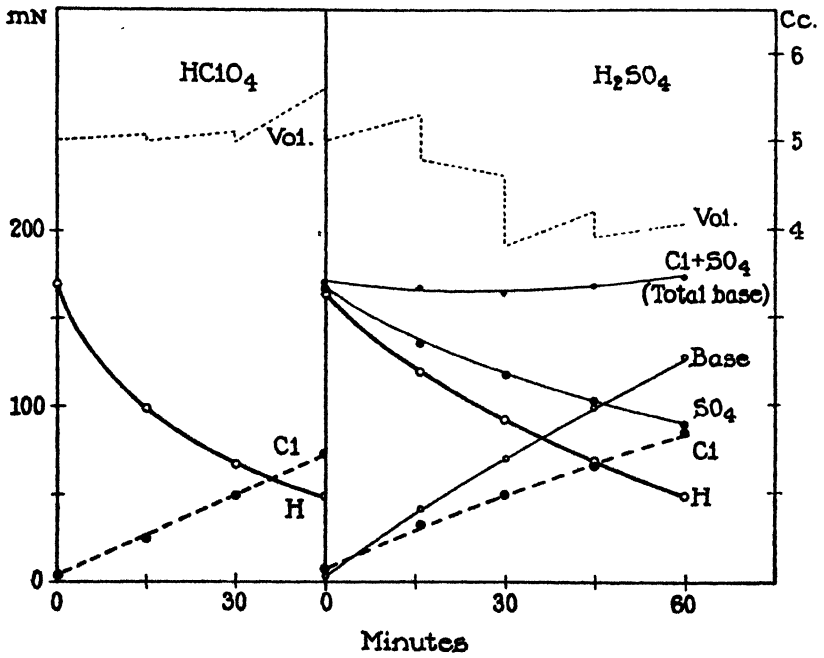

FIG. 3. Introduction experiments with perchloric and sulfuric acid.

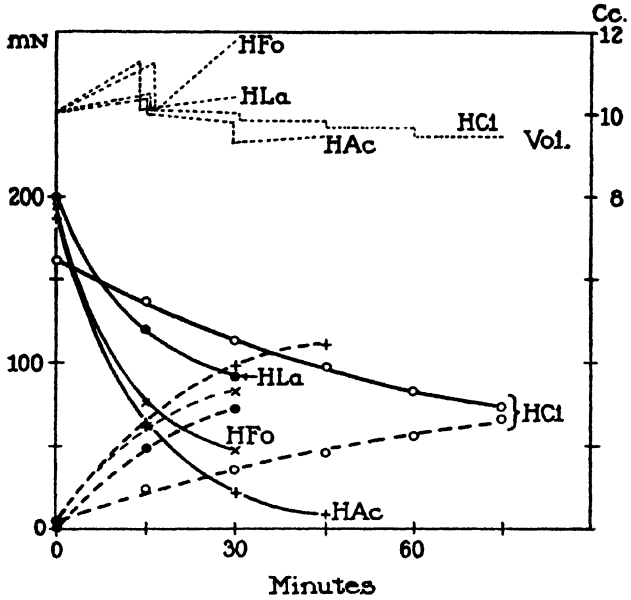

FIG. 4. Introduction of a strong and some weak acids.

—— = acidity ------ = chloride ○ = HCl

● = lactic acid × = formic acid + = acetic acid

The weaker the acid the faster is the decrease in the acidity. Note also the rapid chloride increase with the weak acids.

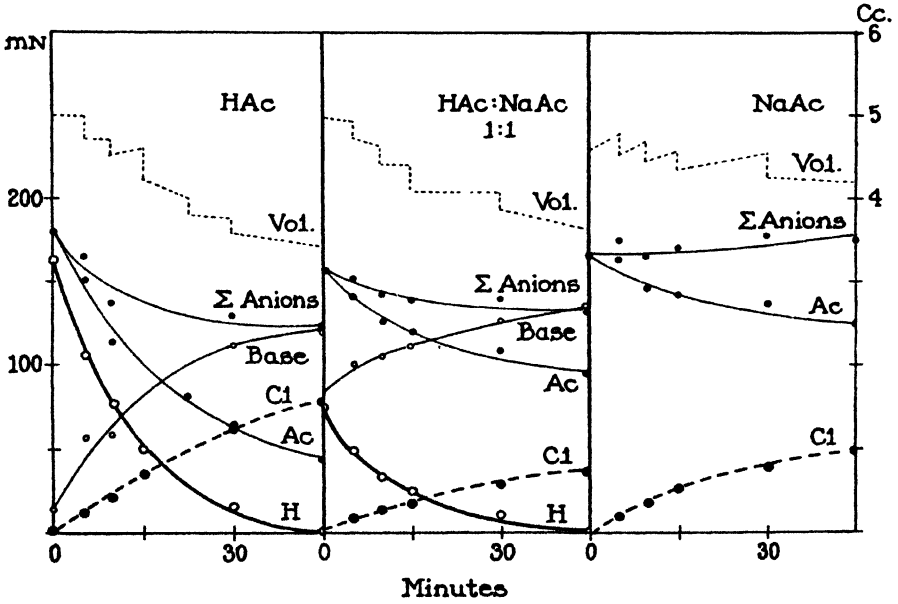

FIG. 5. Introduction of mixtures of acetic acid + sodium acetate. The acetate disappears most rapidly in the undissociated state (*cf.* the HAc curve).

total acetate concentration was about the same in each of these experiments (160–180 mN). (*a*) Pure HAc: The titratable acidity sank very rapidly, also the Ac fell off at a high rate and simultaneously the Cl increased quickly. The sum of anions (= total base) decreased to about 125 mN, which probably represents a slight hypotonicity, a tendency to volume decrease being noticed. (*b*) HAc/NaAc 1:1: Rapid H decrease, much slower Ac decrease,

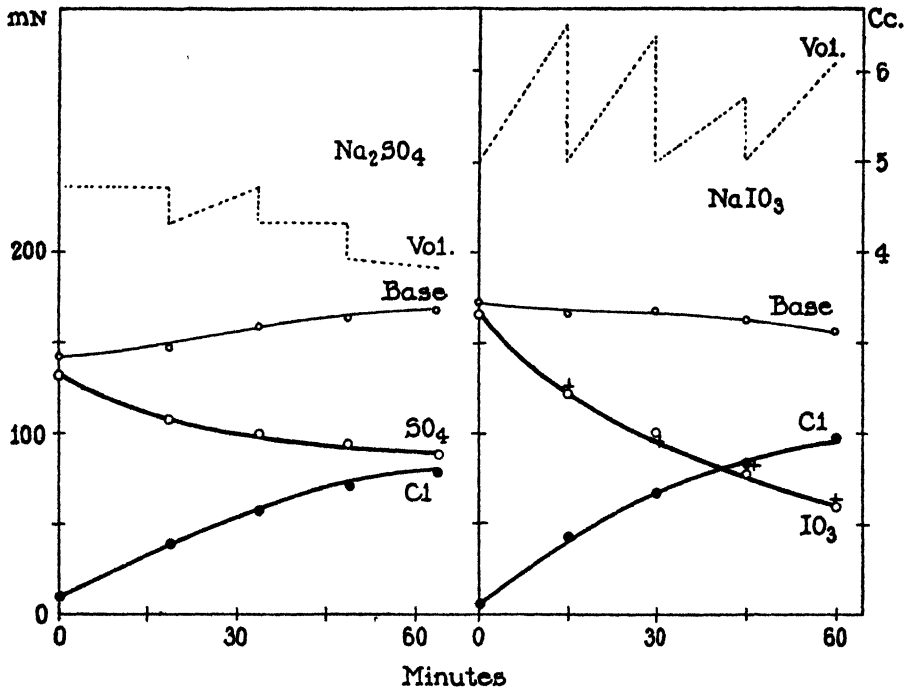

FIG. 6. Introduction of Na-sulfate and Na-iodate. The sulfate disappears very slowly, the corresponding volume changes are insignificant. Scarcely any iodate seems to penetrate the stomach mucosa, as shown by the positions of the crosses denoting the iodate concentration as a result of the successive dilutions (observe the great volume increments).

and a slower Cl increase as compared with HAc alone. Less marked reduction of total anions, insignificant volume variations. (*c*) NaAc: Still slower Ac decrease, slow Cl increase, sum of anions about constant, and a tendency to volume augmentation.

Sodium Sulfate, Na_2SO_4, (Fig. 6). This substance represents a neutral salt. While it was observed that SO_4 concentration diminished slowly, this fact could not be ascribed to dilution, because the volume increment

was negligible. As usual, a steady increase of Cl was observed. The total base increased slowly towards values representing blood isotonicity.

Sodium Iodate, $NaIO_3$, (Fig. 6). This substance, although neutral, showed a behavior which differed markedly from what has been hitherto reported. Large volume increments were observed and when due attention was paid to this circumstance, it was found that the decrease of IO_3 con-

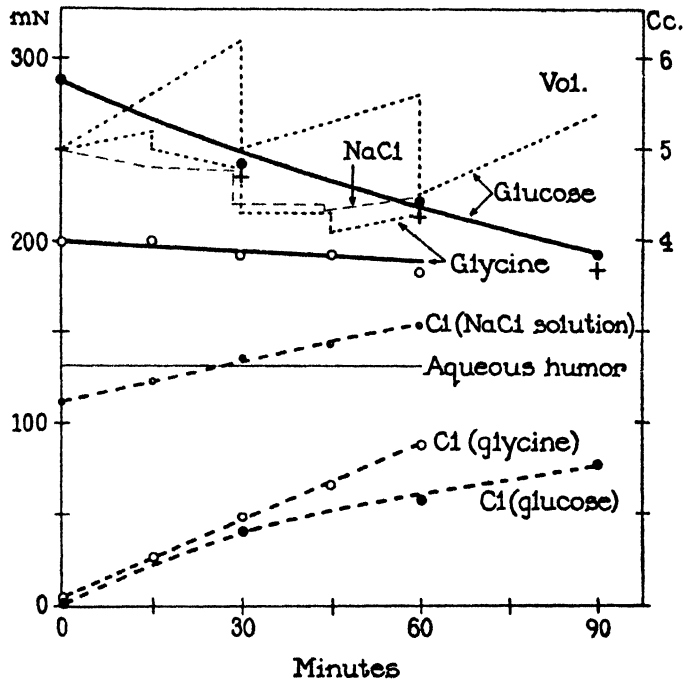

FIG. 7. (*a*) Introduction of glycine and glucose. Insignificant penetration of glycine. The decrease of the glucose concentration is apparently caused by simple dilution (*cf.* the crosses which denote concentrations as calculated from successive dilutions; note the marked volume increments). (*b*) Introduction of a hypotonic NaCl solution. The chlorides rise towards a value higher than the plasma Cl (*ca.* 110 mN) and the Donnan level (*ca.* 130 mN, see "Aqueous humor"). This Cl increase is not accompanied by any volume change.

centration could be fully explained as a dilution effect (compare the crosses and circles in the figure). The Cl values grew more rapidly than usual.

Glucose and Glycine (glycocoll), (Fig. 7). Glucose, although slightly hypotonic (0.3 normal, undissociated), behaved as the iodate, *i.e.* no actual disappearance of the substance out of the stomach cavity was detectable if allowance was made for the dilution caused by the volume increments

(*cf.* cross-marked points in the figure). The glycine concentration, as well as its sample volume, remained practically unaffected. In both cases the usual Cl increase was present.

DISCUSSION

1. The introduced solutions were calculated to be approximately isotonic with the body fluids. Therefore, if the stomach mucosa behaves as a dialysis membrane,[2] solutions of fully permeable substances should not undergo volume changes, but would show concentration changes due to diffusion[3] (group A). Solutions containing poorly permeable substances (group B), on the other hand, might attract water, because the stomach and its content would act as an osmometer (suspended in the mucosa cell fluid or the blood plasma). In the latter case the concentration of the impermeable substance would diminish by dilution only and permeable substances from the mucosa or the blood would increase in concentration owing to diffusion. The substances investigated can be referred to these two groups as follows:

Group A (*Permeable*)	*Group B* (*Poorly permeable*)
Weak acids (HAc, etc.)	$NaIO_3$
Strong acids (HCl, $HClO_4$, etc.)	Glucose
KBr*	Glycine†
$NaHCO_3$*	
NaAc	
Na_2SO_4	

* Footnote 4.
† Footnote 5.

The most striking observation is that the hydrogen ions of strong acids pass out of the stomach contents quite easily. Incidentally, it may be pointed out that the poor permeability and strong water-attracting power of the iodate also show up in model diffusion experiments with cellophane or collodion membranes.

2. The acidity reduction of the strong acids needs a more complete

[2] The stomach content is the "inside" solution; the mucosa cells, or rather the blood, the "outside" solution.

[3] When the substance penetrates much faster than the solutes of the "blood" side one may anticipate a temporary hypotonicity and hence a volume decrease (*cf.* the comments on pure HAc on page 268).

[4] Cited from Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, Fig. 13, p. 297.

[5] The absence of volume increase in the present experiment can probably be ascribed to the fact that the glycine solution was only about "half" isotonic (freezing point—0.28°).

discussion. In general, acid concentration changes might be caused by the following processes; either single or combined:

(*a*) Neutralization of the hydrogen ions by substances acting as bases: alkali bicarbonate, acid-binding mucus, proteins, phosphates, etc.

Against the probability of this process there is, in the first place, the constancy of the introduced volumes. Any neutralizing agent ought to be produced in the dissolved state, and would therefore increase the total volume. A simple calculation is illuminating in this respect: suppose that a hypothetical neutralizing secretion has an acid combining power equal to 0.17 N NaOH (an absurdly high figure). This strength corresponds to that of the freshly secreted gastric hydrochloric acid and to that of the "artificial" acids introduced in these experiments, therefore any volume of acid ought to require an equal volume of the hypothetical secretion in order to be completely neutralized, and hence the total volume would be doubled.

Such an increment was, however, never observed here. One might perhaps argue that a volume increase could have been obscured by a simultaneous "reabsorption" of water from the gastric content. Such a process, however, does not seem probable.

As a further argument against neutralization one can point out that reaction products resulting from a neutralization reaction cannot be recovered in the gastric content in amounts equivalent to the magnitude of the hydrogen ion disappearance. For instance, the "bicarbonate neutralization hypothesis" advanced by Hollander in 1932 has as a consequence the production of carbon dioxide:

$$HCl + NaHCO_3 = NaCl + H_2O + CO_2$$

Introduction of HCl combined with carbon dioxide determinations, however, have failed to demonstrate any significant CO_2 production (Teorell, 1933[6]) during the acidity reduction. Ihre has recently performed quantitative mucus and nitrogen determinations, and he also failed to find a mucus or protein increase corresponding to more than a fraction of the total acidity decrease.

(*b*) Dilution has repeatedly been proposed as a mechanism for the acidity "regulation" (see the review by Hollander[7]). On grounds similar to those that were discussed in the foregoing section on neutralization, one can reject the idea that the reductions of hydrogen ion concentration were caused by simple dilution, the volume changes being too small.

(*c*) Ionic exchange (exchange diffusion) remains as the only mechanism

[6] Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, 288.

[7] Hollander, F., *Am. J. Digest. Dis.*, 1938, **5**, 369.

which can satisfactorily explain the behavior of the acids. The sketch (Fig. 8) may illustrate the exchange diffusion of the hydrochloric acid which is secreted by the stomach itself, and the same exchange is regarded as taking place with artificially introduced HCl. The chief blood constituents which can serve as substitutes for stomach content (gastric juice) constituents are sodium and chloride ions.

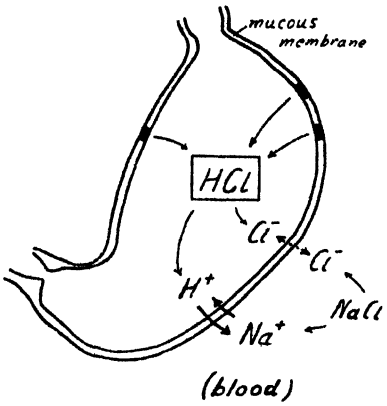

FIG. 8. Sketch schematically explaining the "diffusion theory." The gastric juice is secreted mainly as pure HCl (about isotonic with blood). There is a continuous outward diffusion of HCl and a simultaneous inward diffusion of NaCl—strictly speaking an ionic exchange between H^+ and Na^+. The reduction of acidity and variation of amount of chloride take place in this manner. The larger the volume of liquid, the less is the drop in acidity in unit time. Large volumes are met with when the secretion is rapid, or when the content is increased; for example, owing to introduction of a test meal. The NaCl diffusion can take place even into the contents of a resting stomach (from Teorell, 1935).

3. If the diffusion concepts are correct one might expect that model experiments with artificial diffusion membranes should exhibit similar features as observed in the living animal experiments. Such a similarity has actually been demonstrated by the author.[8] As a further example it may be noted that the characteristic "temporary chloride decrease" displayed in the HCl experiment of Fig. 1, can be found also in a model experiment in which HCl was placed in a cellophane or collodion bag immersed in a large volume of NaCl solution.[9] Also the order of the diffusion rate of different ions or salts is, on the whole, the same in model experiments and in living stomach experiments.

4. As an argument against simple exchange diffusion might appear the fact that the chloride increase which is noticeable in all experiments tends to attain not the level of the blood (or mucosa cell) chlorides of around 100 mN, but a decidedly higher level of about 150 mN (Fig. 7, the experiment with 110 mN NaCl solution).[10] This observation is, however, not against the concept of diffusion since it should be remembered that when dealing with diffusion of ions one has to consider an extra "driving force" besides the osmotic pressure (= concentration difference), namely an electrical

[8] Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, 284–292.
[9] Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, Fig. 11, p. 291.
[10] See also Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, Fig. 12, p. 297.

potential difference that might exist across the diffusion boundary (*cf.* the Donnan effect and the "diffusion effect," Teorell 1935 *b*, 1937). The "accumulation" of Cl ions in the cats' aqueous humor (Fig. 7) to a value exceeding the blood plasma level by *ca.* 30 mN is in all probability a more or less genuine Donnan effect (Bolam, 1932).[11] It is conceivable that the tendency to a chloride accumulation in the stomach is due to a similar mechanism, involving the effect of an electrical "membrane potential."

5. An observation which requires special consideration is the extreme rapidity with which weak acids such as HAc disappeared from the stomach content (Fig. 4). The weaker the acid, the faster it disappeared. This phenomenon could be accounted for by assuming that the undissociated acid molecules could permeate the mucosa by other paths than could the ions, owing to the solubility of these molecules in the non-aqueous parts of the cell membranes. As further evidence may be taken the observation that the pure acid disappeared faster than its salt (Fig. 5). The permeation of weak acids would thus correspond to the mechanism proposed by Osterhout and collaborators (*cf.* Jacques) for the entrance of H_2S and other substances into *Nitella* and *Valonia* plant cells (*cf.* also Maizel's views on the permeability of red blood cells for weak acids). Another reason for an accelerated diffusion can also be suggested. By direct inspection of the opened stomach it can be observed that an acid, as HAc, acts as an "irritant;" the normally feebly pink mucosa acquiring a deeper red color indicating an increased blood flow. It seems justified to assume that this augmented flow of blood results in an increased intensity of diffusion. As evidence of this may be taken the finding that the chlorides in the stomach content increased at double or threefold the "normal" rate in the cases where weak acids were employed (Fig. 4).

6. In conclusion one may state that the cat's stomach mucosa on the whole exhibits properties similar to artificial dialysis membranes, and that therefore the ion concentration variations displayed in stomach contents can be ascribed to pure diffusion processes.

The diffusion hypothesis applied to the gastric hydrochloric acid is capable of explaining the well known parallelism of the gastric juice acidity with the rate of secretion. It can also suggest a mechanism for the total chloride variations discussed by Hollander (1932). It does not seem necessary to assume the existence of any special diluting or neutralizing secretion in order to form a probable picture of the mechanism of the "acidity regulation" and the total chloride variation (*cf.* the discussions by Teorell (1933).[12] However, it should be admitted that in many cases a certain

[11] Bolam, T. R., The Donnan equilibria, London, C. Bell and Sons, Ltd., 1932, 94.
[12] Teorell, T., *Skand. Arch. Physiol.*, 1933, **66**, 292–295.

degree of influence from a diluting and slightly neutralizing "mucus" secretion may exist, particularly in human subjects, when the acid gastric volumes are small, *i.e.* in the beginning and at the end of a secretion period and in pathological cases with low secretion rates (*cf.* Ihre). As a rule, however, it is claimed that "exchange" or "back diffusion" of the hydrochloric acid (hydrogen ions) constitutes the main mechanism of the so called acidity regulation in the stomach.

These diffusion concepts have already been developed qualitatively in earlier papers by the author (1933, 1935) and will be taken up for a further quantitative discussion in a forthcoming paper.

SUMMARY

1. Solutions approximately isotonic with blood of strong and weak acids, several salts, glucose, and glycine were introduced in the resting stomachs of cats. The concentration and volume changes were recorded.

2. It was found that the stomach mucosa was permeable to the majority of the ions tested. There was also a permeability in the opposite direction from the blood (mucosa) to the stomach content, particularly of alkali chlorides. Poorly permeable substances were glucose, glycine, and sodium iodate. Pure weak acids such as acetic acid penetrated very rapidly.

3. The electrolyte concentration changes in the stomach content (or gastric juice) are pictured as an exchange diffusion; for instance, the hydrogen ions of an acid are exchanged against alkali ions of the mucosa or blood.

4. It is pointed out that the concept of the mucosa as an ion permeable membrane could be used as the foundation of a "diffusion theory," which can explain the acidity and chloride variations of the gastric juice without postulating neutralizing or diluting secretions.

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PHYSICOCHEMICAL PROPERTIES OF THE PROTEOLYTIC ENZYME FROM THE LATEX OF THE MILKWEED, ASCLEPIAS SPECIOSA TORR. SOME COMPARISONS WITH OTHER PROTEASES

I. Chemical Properties, Activation-Inhibition, pH-Activity, and Temperature-Activity Curves

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(Received for publication, October 9, 1939)

INTRODUCTION

Proteolytic enzymes have been found in both dicotyledonous and monocotyledonous plants. Representatives of the former are the fig (1), papaya (2), squash (3), legumes, cabbage, and *Euphorbia* (4); of the latter the pineapple (3) and cereals (5). At present it is not definitely known whether these enzymes are all of the same nature, or if each family, genus, or species produces its characteristic variety. Many plant proteases resemble each other in their strong milk clotting power, and in most cases exhibit essentially the same behavior toward oxidizing and reducing agents, *i.e.* they are reversibly inactivated by mild oxidation, and attain their maximal activity in the presence of reducing substances.

The present investigation deals with the proteolytic enzyme from the milkweed, *Asclepias speciosa*, and seeks to establish some distinctive criteria for comparing this enzyme with other plant proteases. This new protease has been named asclepain by the authors. Isolated in a crude but very active form from the fresh latex of the above plant, the enzyme resembles papain in many respects, but shows interesting differences in others. For a substantial study of chemical relationships, it is most desirable to work with crystalline enzymes. However, much valuable information can be gained even with less pure preparations if quantitative methods for measuring protease activity are employed under accurately controlled conditions.

* The material of this paper was taken from a thesis submitted by Theodore Winnick to the Graduate Division in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Isolation of Asclepain

The milkweed from which asclepain was obtained grew on the University of California campus during the summer of 1938. By severing the leaf petioles, the latex which exuded from the cut ends could be collected. This latex was diluted with an equal volume of water, and the suspended particles removed by filtration. The clear filtrate, which contained all the active protease, was titrated with alkali to pH 7.0, and then made up roughly to two-thirds saturation with solid $(NH_4)_2SO_4$, adding 45 gm. of the latter per 100 ml. of solution. The salted out enzyme was collected on a Buchner filter, washed first with 0.5 saturated $(NH_4)_2SO_4$, and then with a small amount of pure water; lastly, the preparation was dried in a vacuum desiccator over H_2SO_4. It was stored in a cold room at $-18°$. Under these conditions, the enzyme retained constant proteolytic activity over a period of many months. 100 ml. of latex yielded 1.5 gm. of faintly yellow material which was used throughout the following experiments.

Materials and Experimental Methods

Asclepain Solutions.—To prepare these solutions, accurate weights of dry enzyme preparation were ground with water or buffer solutions, and diluted to the desired degree. Activators were only used when so specified. The solutions were always prepared within an hour or two of usage.

Proteins.—The proteins used as substrates were hemoglobin prepared according to Anson (6), Van Slyke casein (7), Merck's egg albumin purified by dialysis, edestin prepared according to Osborne (8), and ovovitellin prepared by Jukes[1] (9).

Aqueous Protein Substrates.—These substrates were prepared by grinding weighed amounts of the different proteins with the proper volume of buffer to give solutions of the desired concentrations. The solutions were adjusted with dilute acid or base to the exact pH desired, using the glass electrode to determine the latter. In the cases of edestin and ovovitellin, it was necessary to use buffered 10 per cent NaCl solution as the solvent; even so these proteins were only partly in solution. With the exceptions of hemoglobin and ovalbumin, the proteins were rather insoluble near their isoelectric points, so that the substrates were largely in suspension in this pH region.

Urea Solutions of Proteins.—The technique of Anson (6) was followed in preparing these solutions, except that they were adjusted to the required pH as before. In cases where the substrate is not specified, it will be understood that 2 per cent hemoglobin in 40 per cent urea solution, buffered at pH 7.0, was used.

Methods for Measuring Proteolytic Activity.—Northrop's N.P.N.[2] method (10), Anson's hemoglobin method (6), and the milk clotting method of Balls and Hoover (11), were used in this study. Northrop points out that the N.P.N. method measures principally the primary hydrolysis of the protein, whereas the formol titration, which determines the increase in amino groups, measures chiefly the later stages of digestion. Anson likewise finds that the increase in phenol groups is a good measure of the first stages of proteolysis. The hemoglobin method was used to measure the digestion of both aqueous and urea solutions of the different proteins, and is usually referred to as the "tyrosine color" method in this paper.

[1] A sample of this protein was kindly furnished by Dr. T. H. Jukes of the Poultry Husbandry Division of the University of California.

[2] This abbreviation is used for non-protein nitrogen.

General Properties of Asclepain

Protein Properties.—The dry asclepain preparation dissolved readily in water to give a faintly opalescent solution. There was only a slight turbidity on the addition of $BaCl_2$ solution, proving the virtual absence of $(NH_4)_2SO_4$. The enzyme solution gave the following positive protein color reactions: Millon's, biuret, xanthoproteic, and Adamkiewicz's. A positive test was also obtained for alkali-labile sulfur. The dry material was found by micro-Kjeldahl analysis to contain 16.5 per cent nitrogen.

To prove definitely that the asclepain molecule is of colloidal dimensions, an aqueous solution of the enzyme was placed in a collodion bag and subjected to ultrafiltration. A comparison of the protease activity of the ultrafiltrate with that of the solution remaining in the bag indicated that none of the enzyme had passed through the collodion membrane.

Blood Clotting Test.—Asclepain appeared to differ qualitatively from papain in that it failed to clot citrated or oxalated blood plasma, which was

TABLE I

pH of asclepain solution	1	3.2*	7.0	10.0	13
Tyrosine in 6 ml. digestion mixture after 10 min. at 40° (m.-eq. × 10^3)†	0	3.05	7.5	1.75	0.15

* Asclepain was insoluble at this pH. Probably the latter is near the isoelectric point of the enzyme.

† M.-eq. is used as the abbreviation for milliequivalents.

clotted by papain. According to Eagle and Harris (12), papain converts fibrinogen directly to fibrin in the virtual absence of calcium. Possibly this enzyme has a special group, necessary for blood clotting, which asclepain lacks.

Stability of Asclepain in Solutions of Different pH.—Solutions of different pH, containing 2 mg. of asclepain per ml., were allowed to stand overnight at about 15°. The solutions were then brought to pH 7.0 with dilute acid or base, and diluted exactly twofold in this process. The proteolytic activity of the solutions was then compared by the hemoglobin method. The relative stabilities of the enzyme at the different pH values are indicated in Table I. The results show the enzyme to be most stable in neutral solution.

Rate of Loss of Activity of Papain and Asclepain Solutions on Ageing

Asclepain was found to lose activity slowly on prolonged standing in aqueous or 6.6 M urea solution. The rates of inactivation at 25° and 3° were determined by sampling the ageing solutions at suitable intervals, and

measuring the proteolytic activity by the hemoglobin method. The rate of loss of activity of papain in 6.6 M urea was also measured. The results plotted in Fig. 1 showed that asclepain was inactivated at room temperature at about the same rate in either aqueous or 6.6 M urea solution, and lost about half its initial activity in 30 hours. Papain in 6.6 M urea was inactivated more slowly, and at room temperature lost only about a fourth of

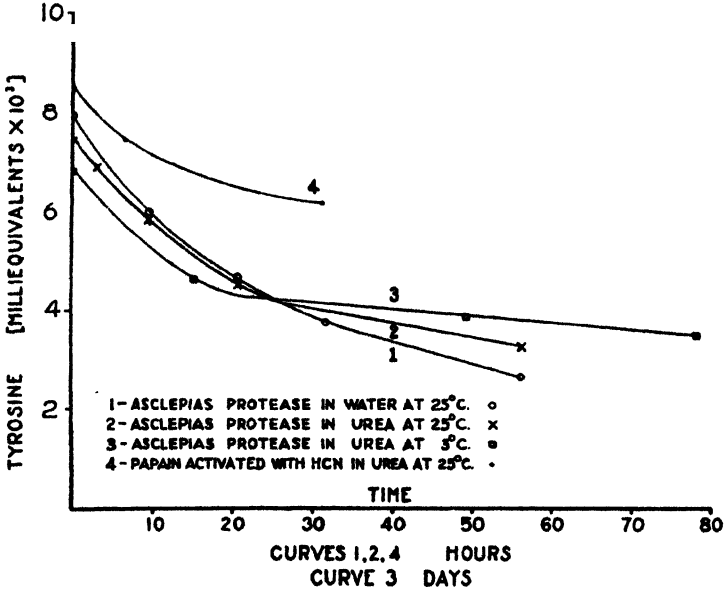

FIG. 1. Decrease in protease activity with ageing of solutions for papain and *Asclepias* protease.

Composition of the enzyme solutions: Curve 1, 1 mg. asclepain per ml. of water; Curves 2 and 3, 1 mg. asclepain per ml. of 6.6 M urea buffered at pH 7; Curve 4, 0.66 mg. Merck's papain per ml. of 0.05 M NaCN and in 6.6 M urea solution. The digestion time and temperature were 10 minutes and 40° in each case.

its activity in 30 hours. At 3°, asclepain lost a fourth of its activity only after about 15 days.

Dialysis of a Urea Solution of Asclepain.—A solution containing 3 mg. of asclepain per ml. of 6.6 M urea, after 10 hours standing at 3°, was placed in a cellophane bag, and dialyzed against pure water in order to remove the urea. The water was changed frequently during a 2 day period. After this time, the white precipitate which had deposited in the bag during dialysis was removed by filtration, and the proteolytic activity of the filtrate was compared with that of an aqueous solution containing 3 mg. asclepain per ml., which had stood at 3° for the same length of time. Nitrogen analyses by micro-Kjeldahl were also made on the two solutions. The results

showed that the dialyzed solution retained about half its initial proteolytic activity, while the nitrogen content was only about a sixth of the original value. This suggests that the asclepain preparation is a mixture of proteins.

Activation and Inhibition of Asclepain

In their comprehensive study of papain activity as influenced by oxidation-reduction and by metal compounds, Hellerman and Perkins (13) found that their results were best explained by the assumption that sulfhydryl is an active grouping in papain, and that proteolytic power is lost when this group is oxidized or when it reacts to form a mercaptide. This study supports the theory first advanced by Bersin and Longemann (14, 15) which postulates that the activation of papain is due to the reduction of dithiol groups in the enzyme molecule.[3] Other plant proteases which resemble papain in their activation and inhibition effects include those of the pineapple (3), fig (1), wheat (5), and *Calotropis* (16). On the other hand sulfhydryl apparently is not essential for the hydrolytic activity of the protease of pumpkin (3) and of several bacterial proteases (17, 18).

In order to compare asclepain with papain, a study was made of the effect on asclepain of a variety of reagents whose activating or inhibiting action on papain was known.

Experimental Technique.—One half ml. portions of asclepain solution containing 3 mg. enzyme per ml. water were treated with specified amounts of different activator and inhibitor solutions. The final volume of each solution was brought to exactly 1.5 ml., adding pure water where further dilution was necessary. Each reagent was allowed to act on the enzyme solution for about a half hour before the activity of the latter was determined. The N.P.N. method was used for most of the measurements. 5 ml. samples of 4 per cent casein solution (buffered at pH 7.5) were digested 40 minutes at 40° by 1 ml. portions of enzyme solution (containing 1 mg. asclepain). The control analyses served to correct for the nitrogen of the activators in cases where this factor was appreciable. In some cases the milk clotting method was also used for comparing proteolytic activities. The hemoglobin method was not suitable for this study, since the phenol reagent gives a blue color in alkaline solution with the sulfhydryl compounds which were used as activators.[4]

The activities of the treated asclepain solutions were compared in all cases with that of the untreated enzyme solution, diluted exactly threefold with pure water. Solutions of cysteine-HCl, thioglycolic acid, iodoacetic acid, NaCN, and Na_2S were always adjusted to pH 7 before being used.

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[3] Balls and Lineweaver (2) believe crystalline papain contains active —SH groups, although the nitroprusside and porphyrindin tests were negative..

[4] Cyanide does not react with the phenol reagent and can be used to activate enzymes for the tyrosine color method.

Interpretation of Results.—The results of the activation and inhibition experiments are reported in Tables II to V. As in the case of papain, the effects with asclepain may be explained by the assumption that a sulfhydryl group in the enzyme molecule is necessary for proteolytic action. The

TABLE II

Activation of Asclepain by Reducing Agents

Activator	Concentration of activator in enzyme solution	N.P.N. in 6 ml. digestion mixture	Ratio of activity to that of untreated enzyme
	M	m.-eq.	
None (untreated enzyme)		0.200	1.00
Cysteine	0.021	0.740	3.70
	0.016	0.750	3.75
	0.008	0.650	3.25
	0.0016	0.400	2.00
	0.0002	0.270	1.35
Sodium cyanide*	0.07	0.665	3.30
Hydrogen cyanide	0.03	0.645	3.25
	0.007	0.465	2.35
Hydrogen sulfide†	0.07	0.450	2.25
	0.035	0.480	2.40
	0.007	0.445	2.25
	0.0015	0.240	1.20
Sodium sulfide	0.05	0.475	2.40
	0.01	0.470	2.35
Sodium thioglycolate	0.03	0.405	2.05
	0.01	0.360	1.80
Sodium bisulfite	0.003	0.325	1.60

* The enzyme solution was treated 3 minutes with alkaline NaCN and then adjusted to pH 7.

† Dilutions were made from a saturated H_2S solution (about 0.1 M).

maximum activation of both asclepain (Table II) and papain (13) is produced by the addition of cysteine. Other reducing agents activate to a lesser degree. With increasing amounts of each activator, the degree of activation rises and attains a maximum, not increased by still more activator. Hellerman and Perkins have advanced explanations for the divergent degree of activation of papain by cysteine, HCN, and H_2S on the basis of

different reaction rates and different modes of chemical attack on the sulfhydryl of the enzyme molecule.

Except when very dilute, H_2O_2 and iodine inactivate asclepain irreversibly (Tables III and IV). This resembles the behavior of papain. Bergmann and Zervas (19) also failed to obtain complete reactivation (of papain) even when very dilute iodine solution was used. According to Purr (20), the reversible inactivation with H_2O_2 is accompanied by a proportional loss of sulfhydryl groups. Presumably higher concentrations of peroxide oxidize the sulfhydryl beyond the dithiol stage.

TABLE III

Inactivation of Asclepain by Hydrogen Peroxide and Reactivation by Cysteine

Volume and concentration of reagent added to 0.5 ml. of enzyme solution		N.P.N. in 6 ml. digestion mixture	Ratio of activity to that of untreated enzyme
Hydrogen peroxide*	Cysteine		
		m.-eq.	
None (untreated enzyme)		0.200	1.00
0.5 ml. of 0.06 M	None	0.005	0.00
Same	0.5 ml. of 0.05 M	0.045	0.20
0.5 ml. of 0.03 M	None	0.000	0.00
Same	0.5 ml. of 0.05 M	0.550	2.75
0.5 ml. of 0.015 M	None	0.010	0.05
Same	0.5 ml. of 0.05 M	0.765	3.80
None	Same	0.775	3.85

* Dilutions were made from 30 per cent hydrogen peroxide (Superoxyl).

TABLE IV

Inactivation of Asclepain by Iodine and Reactivation by Hydrogen Sulfide. Activation by Phenyl Hydrazine

Volume and concentration of reagent added to 0.5 ml. of enzyme solution		Time required for milk clotting	Ratio of activity to that of untreated enzyme*
		min.	
None (untreated enzyme)		6.0	1.0
None	0.25 ml. of 0.1 M H_2S	1.8	3.3
0.25 ml. of 0.003 N I_2	None	†	0.0
Same	0.25 ml. of 0.1 M H_2S	12.5	0.5
0.25 ml. of 0.001 N I_2	None	18.5	0.3
Same	0.25 ml. of 0.1 M H_2S	3.3	1.8
None	0.25 ml. of 0.05 M phenyl hydrazine	2.8	2.1
None	Same plus 0.25 ml. of 0.1 M H_2S	1.9	3.2

* Clotting time is assumed inversely proportional to the amount of active enzyme. This is confirmed in Paper II which deals with the kinetics of milk clotting.

† No clotting occurred after an hour.

In contrast to papain, the inactivation of asclepain by Hg^{++}, Cu^{++}, or Ag^{+} could not be reversed by excess sulfide or cyanide (Table V). Even solid HgS, Ag_2S, and AgBr caused a 70 to 95 per cent inactivation, just as did a concentration of 10^{-4} to 10^{-5} M Ag^{+}. The explanation may be that the

TABLE V

Inactivation of Asclepain by Iodoacetate and by a Variety of Metal Compounds. Attempted Reactivation

Volume and concentration of reagent added to 0.5 ml. of enzyme solution		N.P.N. in 6 ml. of digestion mixture	Ratio of activity to that of untreated enzyme
Inhibitor	Activator		
		m.-eq.	
None (untreated enzyme)		0.200	1.00
0.5 ml. of 0.01 M iodoacetate	None	0.005	0.00
Same	0.5 ml. of 0.1 M H_2S	0.000	0.00
0.25 ml. of 0.01 N Cu^{++}, Hg^{++}, or Ag^{+}	None	0.000–0.005	0.00
Same	0.5 ml. of 0.1 M H_2S, Na_2S, or HCN*	0.000–0.005	0.00
0.2 ml. of 0.01 N Ag^{+}	0.8 ml. of 0.02 N Br*	0.000	0.00
0.1 ml. of 2×10^{-3} N Ag^{+}	None	0.015	0.05
0.1 ml. of 2×10^{-4} N Ag^{+}	None	0.070	0.35
0.1 ml. of 2×10^{-5} N Ag^{+}	None	0.135	0.65
5–10 mg. solid AgBr, Ag_2S, or HgS†	None	0.000–0.060	0.00–0.30
5–10 mg. solid Cu_2O‡	None	0.130	0.65
Same	0.5 ml. of 0.1 M H_2S	0.250	1.25
0.5 ml. of 0.02 N Ni^{++}, Co^{++}, or Zn^{++}	None	0.020–0.030	0.10–0.15
0.5 ml. of 0.02 N Zn^{++}	0.5 ml. of 0.1 M H_2S	0.295	1.45
0.5 ml. of 0.02 N Pb^{++}, Mn^{++}, or Fe^{++}	None	0.090–0.115	0.45–0.55
0.5 ml. of 0.02 N Fe^{+++}	None	0.055	0.25
Same	0.5 ml. of 0.1 M H_2S	0.130	0.65
0.5 ml. of 0.02 N Mg^{++}	None	0.160	0.80

* Resulting precipitate filtered out and the activity of the filtrate determined.

† These precipitates were prepared with an excess of sulfide or bromide and washed thoroughly. They were shaken with the enzyme during a half hour.

‡ Freshly prepared from Fehling's solution and glucose. Shaken with enzyme during a half hour.

enzyme-metal union persists even when the concentration of metal ions in the solution is reduced to the minute values imposed by the solubility products of the above heavy metal salts. Another possibility is that the metal ions catalyze an irreversible oxidation of the enzyme. Harrison (21, 22) has shown that minute amounts of iron and even smaller traces of copper catalyze the aerobic and anaerobic oxidation of sulfhydryl compounds. Sufficient evidence is not at hand to decide from the alternatives.

Also in the cases of Ni^{++}, Co^{++}, Zn^{++}, Pb^{++}, Fe^{++}, Fe^{+++}, and Mn^{++}, the degree of inhibition of asclepain is greater than for papain. None of these ions inactivated the latter enzyme by more than 15 per cent (13).

Like papain, asclepain is irreversibly inactivated by iodoacetate. The work of Dickens (23) may offer a clue to the reaction involved here. This investigator proved that iodoacetate reacts vigorously with sulfhydryl compounds in neutral solution to form products of the type $R—S—CH_2—COO^-$.

The failure of Cu_2O completely to inactivate asclepain is at variance with the theory of sulfhydryl as the active group, since this reagent forms characteristic mercaptides with thiol compounds, and will inhibit papain completely. It is possible that the incomplete inactivation of asclepain is due to the slowness of the reaction under the experimental conditions used.

Asclepain resembles papain in being activated by phenyl hydrazine. Bergmann and Ross (24) in developing their theory for the existence of two enzymes in papain, suggest that phenyl hydrazine causes the dissociation of the proteinase-peptidase complex by reacting with an aldehyde group, thereby liberating the two active enzymes. Hellerman (25) points out that phenyl hydrazine can react in several ways, so that no definite conclusion regarding the mechanism of its action can be drawn. Balls and Hoover (11) find that this reagent accelerates the milk clotting action of papain several fold, and attribute this as presumably being due to a reduction of the natural activators of the papaya.[5]

pH-Activity Relations for Different Proteins Digested by Asclepain

The influence of pH upon the rate of proteolysis has been interpreted in terms of the ionization of the enzyme and of the protein substrate, and numerous studies have dealt with the "optimum pH" of enzymes. Hence it was thought of value to determine the nature of the pH-activity curves for asclepain on several proteins of widely varying character. Both concentrated urea and aqueous buffers were used as solvents, in order to compare the curves of native proteins with those of the same proteins denatured in urea.

Native Proteins.—The pH-activity curve for each of five different proteins was obtained as follows: 5 ml. portions of protein solution at equal concentrations, but buffered at different pH values, were digested for exactly equal times at constant temperature with 1 ml. of the same asclepain solution. The amount of proteolysis was then measured by the tyrosine color method.

[5] Glutathione has been isolated from the fresh fruit of papaya (Ganapathy, C. V., and Sastri, B. N., *Proc. Indian Acad. Sc.*, 1938, **8B**, 399).

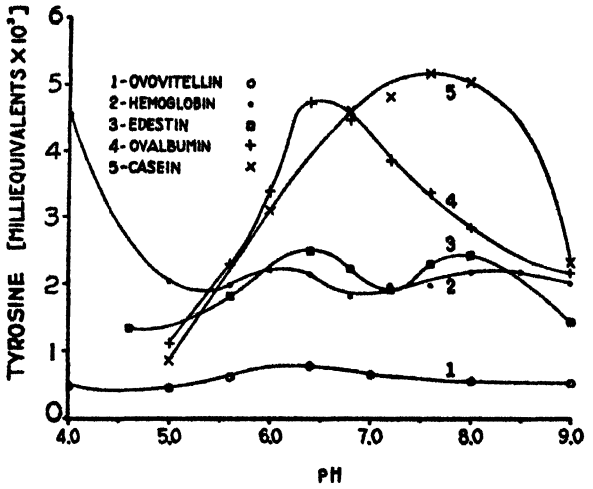

FIG. 2. pH-activity curves of *Asclepias* protease on native proteins.

Curves 1 and 4 represent 2 per cent ovovitellin and ovalbumin, respectively, each digested 60 minutes by 1 mg. enzyme; Curve 2, 3 per cent hemoglobin digested 60 minutes by 2 mg. enzyme; Curve 3, 2 per cent edestin digested 20 minutes by 1 mg. enzyme; Curve 5, 2 per cent casein digested 10 minutes by 1 mg. enzyme.

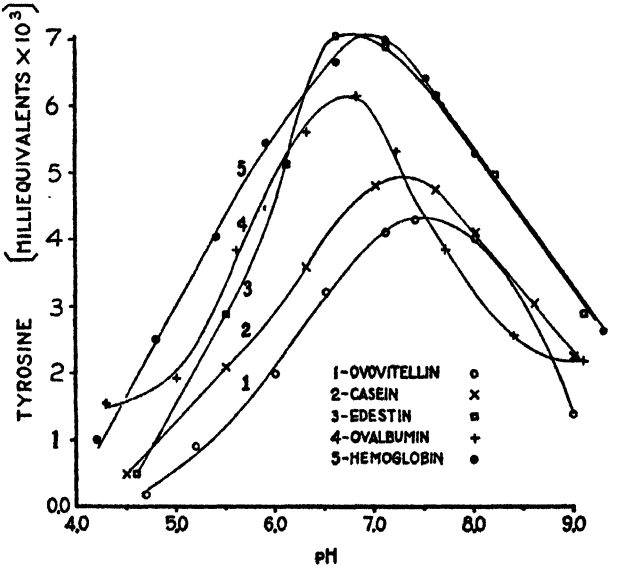

FIG. 3. Activity-pH curves of denatured proteins digested by *Asclepias* protease.

1 mg. enzyme was used for each digestion. The times for the different curves were: 1, 30 minutes; 2, 5 minutes; 3, 4, 5, all 10 minutes.

Fig. 2 shows that the digestibility of the different native proteins varied enormously. Quantitative comparisons are not possible in most cases, since the experimental conditions were necessarily adapted to the digestibility of each of the proteins. It is evident, however, that casein is by far the most digestible. Edestin is moderately digestible, ovalbumin somewhat less so, hemoglobin is only slowly attacked, while ovovitellin is the most difficult of all to digest.

In general, the amount of digestion appears to be least or to go through a minimum in the isoelectric region of each protein.[6] Northrop's theory (26), which relates the rate of proteolysis to the amount of ionized protein, seems applicable to the results with asclepain. Asclepain, like trypsin and unlike papain, seems to act better on the anionic rather than the isoelectric protein. In the case of hemoglobin, the large increase in digestibility in the region of pH 4 to 5 may be due to the partial denaturation of the protein.

Proteins in Urea.- Anson (6) found that such proteins as hemoglobin, ovalbumin, and edestin were rapidly digested in urea solution by proteases. In view of the remarkable solvent properties and denaturing action of this agent, it was of interest to determine the character of the pH-activity curves of the above proteins in 6.6 M urea solution, using the same procedure as before.

The curves of the proteins in urea, given in Fig. 3, are seen to be very similar, with maxima in the vicinity of pH 7.0. The amount of digestion does not appear related to the degree of ionization of the proteins.[7] Edestin, ovalbumin, and hemoglobin, which are moderately or poorly digestible in water, are digested about as vigorously as casein when dissolved in urea. Ovovitellin is less digestible than the other proteins, but is still much more digestible in urea than in aqueous solution.

Effect of Temperature on the Rate of Protein Digestion by Asclepain

The comparison of digestion rates at different temperatures can give information concerning both the temperature coefficients of the reaction and the coefficients of inactivation of the enzyme. In order to study these factors in the case of asclepain, several sets of protein digestions at varying

[6] Hitchcock, D. I., Amphoteric properties of amino acids and proteins, in Schmidt, C. L. A., Chemistry of the amino acids and proteins, Springfield, Illinois, C. C. Thomas, 1938, 618, gives the following pI values: oxyhemoglobin, 6.7; casein, 4.6; ovalbumin, 4.8. The literature reports values for edestin ranging from pI 5.5 to 7.0.

[7] Burk and Greenberg (27) have shown that the isoelectric points of casein and hemoglobin are only slightly different in 6.6 M urea solution than in water.

temperatures were conducted with this enzyme. For each series, 5 ml. samples of protein solution, buffered at the optimum pH, were digested for equal times by the same amount of enzyme. The results are plotted in Fig. 4. The peak of each curve (the greatest amount of digestion) corresponds to the optimum temperature.

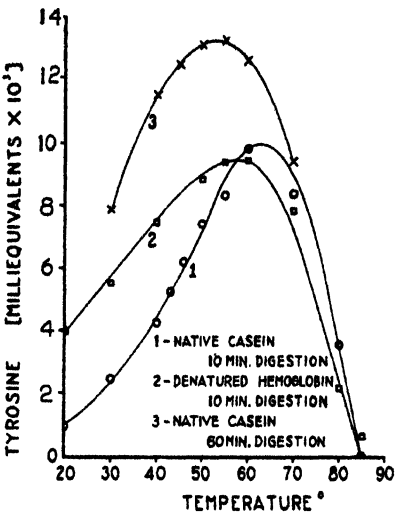

FIG. 4

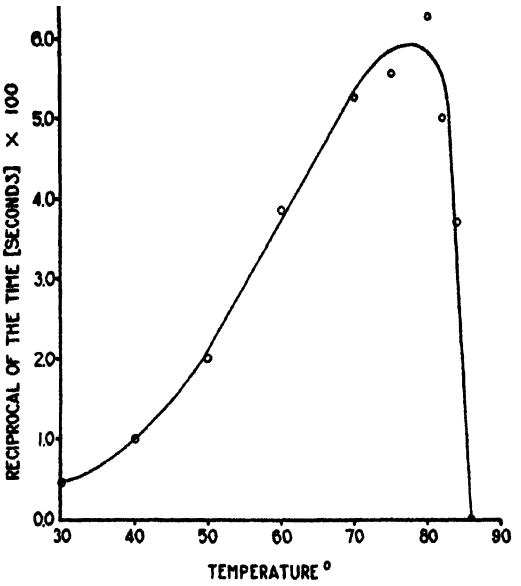

FIG. 5

FIG. 4. Temperature-activity curves of *Asclepias* protease for casein and hemoglobin.

1 mg. enzyme was used for each digestion. Curves 1 and 3 represent 10 and 60 minute digestions, respectively, for 2 per cent casein in aqueous buffer at pH 7.5. Curve 2 represents 10 minute digestions of 2 per cent hemoglobin in 6.6 M urea buffered at pH 7.0.

FIG. 5. Temperature-activity curve of *Asclepias* protease on milk clotting.

Substrate: 20 per cent powdered milk, buffered at pH 7.5. Enzyme: Fresh *Asclepias* latex diluted 15 times with water.

In Fig. 5, which represents the influence of temperature on milk clotting, the activity is expressed as the reciprocal of the clotting time, and the optimum temperature corresponds to the minimum time required for clotting.

The optimum temperature is seen to increase as the reaction time decreases. For the 60 minute digestions of casein, the optimum is about 53°. For the 10 minute digestions of casein and hemoglobin, the corresponding optima are approximately 63° and 58°. For milk clotting,

where the required time is less than a minute, the optimum temperature is raised to about 75°. If the 10 minute digestion curves of native casein and denatured hemoglobin are compared, the temperature coefficients of digestion below the optimum temperature are seen to differ considerably. But above about 65°, where the destruction of enzyme is the dominant factor, the proteolysis decreases at the same rate for both proteins. Above 85°, the enzyme is destroyed even before milk clotting can occur.

SUMMARY

1. A study has been made of the properties of a hitherto unreported proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa*. The new protease has been named asclepain by the authors.

2. The results of chemical, diffusion, and denaturation tests indicate that asclepain is a protein.

3. Like papain, asclepain clots milk and digests most proteins, particularly if they are dissolved in concentrated urea solution. Unlike papain, asclepain did not clot blood.

4. The activation and inhibition phenomena of asclepain resemble those of papain, and seem best explained on the assumption that free sulfhydryl in the enzyme is necessary for proteolytic activity. The sulfhydryl of asclepain appears more labile than that of papain.

5. The measurement of pH-activity curves of asclepain on casein, ovalbumin, hemoglobin, edestin, and ovovitellin showed no definite digestion maxima for most of the undenatured proteins, while in urea solution there were well defined maxima near pH 7.0. Native hemoglobin and ovovitellin were especially undigestible, while native casein was rapidly attacked.

6. Temperature-activity curves were determined for asclepain on hemoglobin, casein, and milk solutions. The optimum temperature was shown to increase with decreasing time of digestion.

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PHYSICOCHEMICAL PROPERTIES OF THE PROTEOLYTIC ENZYME FROM THE LATEX OF THE MILKWEED, ASCLEPIAS SPECIOSA TORR. SOME COMPARISONS WITH OTHER PROTEASES

II. Kinetics of Protein Digestion by Asclepain

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INTRODUCTION

Much valuable information concerning enzymes can be obtained by quantitative studies of reaction rates, in which the enzyme concentration, initial substrate concentration, and time are varied. This paper deals with the kinetics of the digestion of hemoglobin in urea, and casein in both urea and aqueous solutions, by the protease of the milkweed, *Asclepias speciosa*. Some of the physicochemical properties of this new enzyme, named asclepain, have been previously described.[1] In the present study, the order of the reaction and the nature of the enzyme-substrate intermediate were investigated for the digestion of hemoglobin and of casein in urea solution by asclepain, and the Schütz-Borissov rule was tested. The kinetics of milk clotting by asclepain were also examined.

Milk Clotting Relations

In their study of the milk clotting action of papain, Balls and Hoover (1) showed that the influence of enzyme concentration on the time required for milk coagulation is quite accurately given by the equation $(E\text{-}c)t = K'$, where E is the weight of enzyme, t the time, and K' a constant. The interpretation of Balls and Hoover is that c represents a constant weight of enzyme which is removed from clotting action by some component in the milk substrate.

This relation was tested for asclepain by the same technique used by Balls and Hoover for papain. Varying dilutions were made from a solution which contained 1.5 mg. asclepain per ml. and 0.05 M cysteine (at pH 7)

[1] Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, 1940, **23**, 275.

for activation. The time required for one part of enzyme solution to clot 10 parts of milk substrate (buffered at pH 7.5) was then determined for each dilution.

The results are given in Fig. 1. The amount of enzyme is plotted against the reciprocal of the clotting time. In accordance with the Balls and Hoover equation, the intercept on the ordinate gives the value $c = 0.08$ mg. When this quantity is substituted in the equation, the K' values obtained range from 1.85 to 2.05. For papain c was somewhat larger (0.27 mg.). In the case of crystalline chymotrypsin (1), apparently none of the enzyme is inhibited, since the plot of E against $1/t$ passes through the origin.

Balls and Hoover define unit clotting activity of an enzyme as the amount which will cause milk to clot in 1 minute under the conditions de-

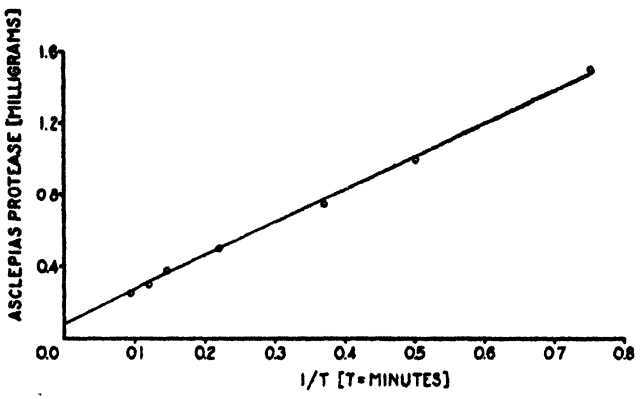

FIG. 1. Effect of *Asclepias* protease concentration on milk clotting time

scribed above. Accordingly, the units per mg. enzyme are equal to $1/E \cdot t = 1/K'$ at higher concentrations, or $1/(E-c)t = 1/K'$ where the amount of enzyme inactivated is significant. From the average value, $K' = 1.9$, for asclepain, 1 mg. of enzyme preparation represents 0.53 clotting units. This value is somewhat higher than those found by Balls and Hoover with crude papain activated by H_2S (0.21 to 0.39 units per mg.).

Evaluation of Asclepain Activity from Enzyme Concentration Curves

The amount of proteolysis produced by different quantities of asclepain in a definite time was measured by Northrop's casein N.P.N. method (2) and Anson's hemoglobin method (3). For each series of digestions, different dilutions from an enzyme solution containing 1 mg. asclepain per ml., activated by 0.05 M NaCN, were used to digest samples of the same protein substrate. The data obtained are given in Table I.

By plotting the amount of enzyme against the quantity of casein digested,[2] the degree of proteolytic activity was compared for the 20 and 40 minute periods. From a line drawn tangent to the first part of each curve (*i.e.* the part which intercepts the origin), the quantities of N.P.N. in 6 ml. digestion mixtures yielded by 1 mg. enzyme were read off. These values were found to be 0.58 and 0.98 m.-eq. per mg. enzyme for the 20 and 40 minute digestions, respectively. Apparently the amount of digestion is not exactly proportional to the digestion time when the latter is of many minutes duration. The activity is, in fact, better evaluated from activity-time curves, as is shown in the next section.

TABLE I

Digestion of Casein in Water and Hemoglobin in Urea at 40° by Different Amounts of Asclepain

Enzyme in 6 ml. digestion mixture	N.P.N. in 6 ml. digestion mixture		Tyrosine in 6 ml. digestion mixture after 10 min.
	20 min.	40 min.	
mg.	*m.-eq.*	*m.-eq.*	*m.-eq.* × 10^3
1.0	0.465	0.680	14.4
0.5	0.295	0.485	11.7
0.33			8.9
0.25	0.150	0.260	6.9
0.165	0.090	0.155	
0.125	0.070	0.120	3.7
0.065			1.6

The aqueous casein substrate contained 0.36 per cent casein nitrogen (about 2–2.5 per cent protein) and was buffered at pH 7.5. The hemoglobin solution was 2 per cent in 6.6 M urea and buffered at pH 7.0.

The degree of proteolytic activity as measured by the hemoglobin method, was evaluated from a plot of tyrosine color against amount of enzyme.[2] Using the initial slope as before, it was found that 0.036 mg. enzyme gave a color value of 0.001 m.-eq. of tyrosine in 6 ml. digestion mixture after a 10 minute digestion. Anson defines one protease unit as "the amount which digests hemoglobin under the standard conditions at an initial rate such that there is liberated per minute an amount of split products not precipitated by trichloracetic acid which gives the same color with the phenol reagent as one milliequivalent of tyrosine." From this definition, one asclepain unit is contained in 0.036 × 1000 × 10 = 360 mg. of the enzyme preparation; or 1 mg. enzyme equals 0.0028 units.

[2] These plots are omitted in order to save space.

Activity-Time Relations for Asclepain on Casein and Hemoglobin

Sets of activity-time relations were obtained by digesting samples of casein and hemoglobin substrates for varying times at 40° with the same amount of enzyme. The results are summarized in Table II.

TABLE II

Digestion of Hemoglobin and Casein at 40° for Varying Lengths of Time by a Definite Amount of Asclepain

Time of digestion	N.P.N. in 6 ml. digestion mixture, m.-eq.		Tyrosine in 6 ml. digestion mixture, m.-eq. × 10^3				
	Aqueous casein substrates at pH 7.6		Solutions in 6.6 M urea				
	0.64 m.-eq. casein N	1.28 m.-eq. casein N	2 per cent casein pH 7.5	4 per cent casein pH 7.5	1 per cent hemoglobin pH 7.0	2 per cent hemoglobin pH 7.0	4 per cent hemoglobin pH 7.0
min.							
2	0.050	0.100					
4	0.095	0.185					
5			2.45	2.90	2.25	3.20	3.85
6	0.145	0.295					
8	0.195	0.340					
10			3.95	4.95	3.45	4.60	5.95
15	0.265			6.20			
20		0.535	6.70	7.50	4.65	6.70	8.85
25				8.45			
30	0.345						
35			9.20	9.90			
40		0.685			5.70	8.45	10.80
45				10.90			
50			10.30				
52	0.400						
60		0.775			6.15	8.90	11.75
70	0.445		11.45				
80		0.870					
85	0.470						

The enzyme solutions used were: 1 mg. asclepain per ml. + 0.02 M cysteine for the aqueous casein solutions; 0.5 mg. asclepain per ml. of water for the casein in urea; 0.165 mg. asclepain per ml. + 0.05 M HCN for the hemoglobin in urea.

The enzyme activity was calculated from the initial slopes of the plots of the amount of digestion against the time.[2] For the aqueous casein substrates, the activity unit of Northrop, *i.e.* the amount of enzyme which liberates N.P.N. soluble in 5 per cent trichloracetic acid at the rate of 1 m.-eq. per minute in the digestion mixture under standard conditions, was employed. Accordingly for the substrates containing 1.28 and 0.64 m.-eq.

casein nitrogen (per 5 ml.), the corresponding activity units per mg. asclepain are 0.0475 and 0.024. It is seen that the initial rate of proteolysis is directly proportional (within the limits of experimental error) to the substrate concentration.

Similarly, from the initial slopes of the curves plotted for casein and hemoglobin in urea,[2] the tyrosine color produced per minute in 6 ml. digestion mixture, was read off. Taking into account the quantities of enzyme used, the activity units per mg., as defined by Anson for hemoglobin, were calculated as was done for the activity-enzyme concentration data. The activity values given in Table III were obtained for the proteins in urea.

It is seen that the initial rates of digestion in urea are not proportional to the substrate concentration. This is more clearly shown by the activity-substrate concentration curves given in the next section. The activity

TABLE III

Substrate	Tyrosine in 6 ml. digestion mixture per minute	Asclepain units per mg. enzyme
	m.-eq. × 10^3	*units* × 10^3
4 per cent hemoglobin	0.80	4.8
2 per cent hemoglobin	0.68	4.1
1 per cent hemoglobin	0.48	2.9
4 per cent casein	0.58	1.15
2 per cent casein	0.48	0.95

values are much higher for the hemoglobin solutions because the activated enzyme was used with this substrate, while non-activated asclepain was used with the casein.

The activity of activated asclepain in the present preparation (in hemoglobin units) was only about a tenth that of crystalline pepsin or trypsin and about half that of crystalline chymotrypsin.[3] In terms of casein N.P.N. units, the asclepain activity was about equal to that of pepsin, but much less than that of trypsin or chymotrypsin.

Kinetics of Digestion.— Rate equations were applied to the data of Table II in order to determine the order of reaction where possible. The calculation of velocity constants for the aqueous casein digestions showed that the first and third order constants fluctuate, while the second order constants

[3] The activity values (taken from Northrop, J. H., Crystalline enzymes. The chemistry of pepsin, trypsin, and bacteriophage, Columbia Biological Series, No. 12, New York, Columbia University Press, 1939) could be expressed in terms of mg. enzyme, since each crystalline enzyme contains about 15 per cent nitrogen.

attain an approximately constant value after 50 to 60 minutes. The calculations suggest that the reaction approaches the second order rate, but follows no order closely or consistently.

It is of interest that Nasset and Greenberg (4) found the rate of hydrolysis of casein by acids to conform to the second order equation. Northrop (5) found that under certain conditions the digestion of casein with crude trypsin followed the course of a first order reaction. With purified trypsin, the first order constant decreased as the reaction proceeded. For denatured (aqueous) hemoglobin, he found that the first order constant decreased with both pure and crude trypsin.

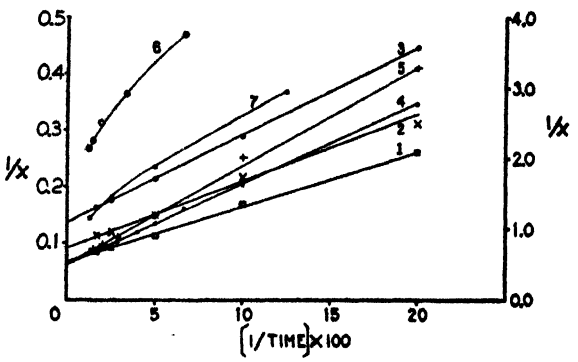

FIG. 2. Kinetics of the hydrolysis of casein and hemoglobin by *Asclepias* protease.

Curves 1, 2, and 3 represent 4, 2, and 1 per cent hemoglobin substrates, respectively, in 6.6 M urea. Curves 4 and 5: 4 and 2 per cent casein substrates, respectively, in 6.6 M urea. Curves 6 and 7: aqueous casein solutions containing 0.64 and 1.28 m.-eq. casein nitrogen per 5 ml., respectively. $1/x$ is expressed in terms of m.-eq. tyrosine $\times 10^3$ for curves 1 to 5 (left ordinate) and by m.-eq. N.P.N. for curves 6 and 7 (right ordinate).

The second order equation may be written in the form

$$\frac{1}{Kt} = \frac{C_0^2}{x} - C_0$$

where C_0 is the initial substrate concentration, x the concentration of product at time, t, and K is the velocity constant. It is seen that the plot of $1/t$ against $1/x$ should give a straight line if the reaction is second order.

In Fig. 2 the reciprocal of the amount of digestion is plotted against the reciprocal of the time $\times$ 100, using the data of Table II. The plots show that the second order equation describes the digestion rates of hemoglobin and casein in urea fairly accurately. The plots for the aqueous casein solutions deviate somewhat from a linear relation. This confirms the conclusion that the digestion here only roughly approaches the second order rate. Plots of ln $(C_0\text{-}x)$ against t (test for first order) do not give straight lines in any case.

Linderstrøm-Lang (6) states that proteases catalyze a bimolecular reaction between peptide bonds and water. However, this does not explain the second order rate in urea solution, since the water is still present in so large

an excess that its mass action effect does not change significantly during the reaction.

From the second order equation it is seen that when $1/x = 0$,

$$C_0 = -\frac{1}{Kt}, \quad \text{or} \quad K = -\frac{1}{C_0 t}.$$

This gives a means for comparing the different velocity constants for the proteins in urea. The curves in Fig. 2 are extrapolated to their points of intersection on the negative $1/t$ axis, and the values of these intercepts, together with the C_0 values,[4] are substituted in the last equation. The velocity constants obtained are given in Table IV. They do not appear accurately proportional to the initial substrate concentrations.

TABLE IV

Substrate	Second order constant	C_0K
4 per cent hemoglobin	0.017	0.068
2 per cent hemoglobin	0.038	0.076
1 per cent hemoglobin	0.087	0.087
4 per cent casein	0.010	0.040
2 per cent casein	0.017	0.034

Activity-Substrate Relations Based on the Michaelis-Menten Theory

Lineweaver and Burk (7) have developed graphic methods for determining dissociation constants of enzyme-substrate compounds when the data are consistent with an assigned mechanism. The general case for the simplest equilibrium between enzyme and substrate may be represented by the equation

$$K_s = \frac{(E)(S)^n}{(ES_n)}.$$

(E) and (ES_n) are the concentrations of free and combined enzyme, respectively, (S) is the concentration of substrate, and n is the number of molecules of substrate which combine with one molecule of enzyme. The equilibrium constant K_s represents the nth power of the substrate concentration at half the limiting velocity.

The Michaelis-Menten equation can then be written in the form

$$\frac{1}{v} = \frac{K_s}{V_m(S)^n} + \frac{1}{V_m}$$

[4] These are simply taken as the percentage protein in each substrate prior to the addition of enzyme. They cannot be expressed in terms of tyrosine color values.

where v is the observed initial reaction velocity and V_m a constant representing the maximum velocity (which theoretically corresponds to infinite substrate concentration).

A plot of $1/v$ against $1/(S)^n$ yields a straight line whose intercept on the

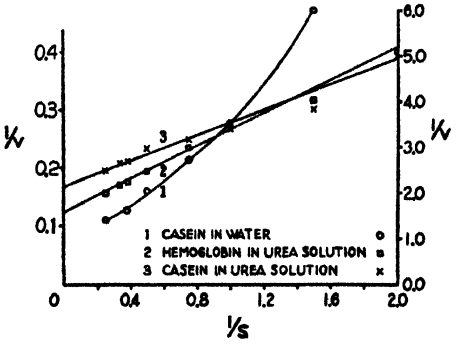

FIG. 3

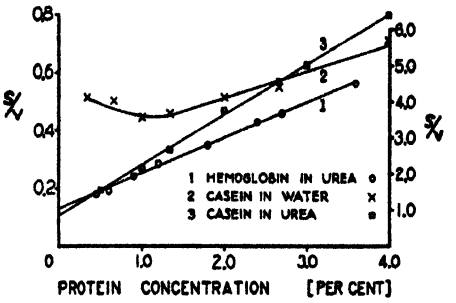

FIG. 4

FIG. 3. Nature of the enzyme-substrate intermediate of *Asclepias* protease with hemoglobin and casein.

The velocity, v, is expressed in m.-eq. tyrosine $\times 10^3$ produced in 6 ml. digestion mixture in 5 minutes for the urea solutions (left ordinate), and in m.-eq. N.P.N. produced in 6 ml. digestion mixture in 20 minutes for the aqueous casein solutions (right ordinate). S is the protein concentration in per cent. All digestions were at 40°. The aqueous casein digests (buffered at pH 7.5) contained 1 mg. enzyme activated with cysteine. The casein and hemoglobin solutions in urea, buffered at pH 7.5 and 7.0 respectively, were digested in each case by 0.25 mg. enzyme (activated by alkaline cyanide).

FIG. 4. Test of enzyme-protein intermediate.

The left ordinate represents the ratio of protein concentration (per cent) to tyrosine color value (m.-eq. $\times 10^3$) for the urea solutions, and right ordinate the ratio of protein concentration (per cent) to N.P.N. (m.-eq.).

$1/v$ axis is $1/V_m$, and whose slope is K_s/V_m. In this way K_s can be at once evaluated. Multiplied through by $(S)^n$, the last equation becomes

$$\frac{(S)^n}{v} = \frac{K_s}{V_m} + \frac{(S)^n}{V_m}.$$

When $(S)^n/v$ is plotted against $(S)^n$, a straight line is again obtained. The intercept on the $(S)^n/v$ axis is K_s/V_m, and the slope is $1/V_m$.

These graphic methods were applied to the digestion of casein and hemoglobin in 6.6 M urea and casein in water, by asclepain. Different dilutions were made from these substrates with the aqueous buffer or the buffered 6.6 M urea used in preparing the initial protein solutions. The

digestion rates for varying initial substrate concentrations were then determined, using the N.P.N. method for the aqueous solutions and the tyrosine color method for the proteins in urea.

In Fig. 3 the reciprocal of the digestion rate is plotted against the reciprocal of the protein concentration in order to determine the nature of the enzyme-substrate compound. The values for casein and hemoglobin in urea fall fairly well along straight lines, while those for casein in water depart from a linear relation. Plots using higher powers of the substrate concentrations do not yield straight lines.[5] One may conclude from the results that a definite enzyme-substrate compound, consisting of one molecule each of enzyme and of substrate, initiates the hydrolysis of casein and hemoglobin by asclepain in urea solution.

In Fig. 4 where the ratio of protein concentration to digestion rate is plotted against protein concentration, the departure of the aqueous casein

TABLE V

Substrate	Enzyme-substrate dissociation constant	
	From plot of $1/v$ against $1/S$	From plot of S/v against S
Casein in urea	0.65	0.60
Hemoglobin in urea	1.10	1.05

data from a straight line is more apparent. The linear relations obtained for the proteins in urea also indicate the absence of substrate inhibition, since in cases where there is an inactive enzyme-substrate compound, plots of $(S)^n/v$ against $(S)^n$ give curves that rise concavely with increasing substrate concentration.

Casein in water apparently fails to form a single definite compound with asclepain. It will be recalled that the digestion rate of this protein did not adhere closely to any reaction order. Northrop (8) was also unable to obtain definite evidence for the existence of an intermediate compound of casein with trypsin in aqueous solution.

Table V gives the values of the enzyme-substrate dissociation constants for asclepain and the proteins in urea, calculated from the graphs in Figs. 3 and 4.

[5] Lineweaver and Burk found that plots of $1/v$ *vs.* $1/S$ gave straight lines for invertase, raffinase, and amylase, while for citric dehydrogenase, a linear relation could only be obtained by plotting $1/v$ *vs.* $1/S$.[2]

Inhibition by Reaction Products—The Schütz-Borissov Rule

As was shown many years ago by Schütz (9) and Borissov (10) for pepsin, the amount of digestion produced by proteases in a given time increases as the square root of the enzyme concentration. This relation may be expressed as

$$K = \frac{x}{\sqrt{Et}}$$

where x is the amount of proteolysis, E the enzyme concentration, and t the time.

Arrhenius (11) showed that this deviation from the equation of simple catalysis could be explained by assuming that the reaction product formed

TABLE VI

Verification of the Schütz-Borissov Rule for Asclepain on Hemoglobin in Urea

Enzyme per 6 ml. digestion mixture E	Time required for production of 16×10^{-3} m.-eq. tyrosine in 6 ml. of digestion mixture	$E \cdot t$
mg.	*min.*	
2.66	6.0	16.0
2.00	8.5	17.0
1.33	13.1	17.5
1.00	17.0	17.0
0.66	26.5	17.5
0.33	62.5	20.5

an inactive compound with the enzyme, in accordance with the mass action law. Northrop (12) showed that pepsin and trypsin were actually inhibited by the products of proteolysis, and that the Schütz-Borissov rule was followed or approximated under suitable conditions, particularly when the concentrations of both substrate and product were large, relative to the enzyme concentration.

Bodansky (13) points out that the statement "the velocity of reaction is proportional to the square root of the enzyme concentration" is based on an incorrect use of the term "velocity of reaction." He states that Schütz's own data show that the velocity is directly proportional to the first power of the enzyme concentration, and that the square root relation holds when the reciprocal of the time for a given change in the substrate is used as a measure of the reaction velocity.

This last approach was used to test the rule for asclepain on hemoglobin

in urea. Using different amounts of enzyme, several sets of digestions were made at 40° and pH 7.0 by the hemoglobin method, with reaction times chosen to give as nearly as possible the same amounts of digestion. From the curves representing digestions with varying amounts of enzyme,[2] the exact times required for the production of the color equivalent of 16×10^{-3} m.-eq. of tyrosine in 6 ml. of digestion mixture filtrate were interpolated. These interpolated times, together with the corresponding amounts of enzyme, are recorded in Table VI. It is seen that the product $E \cdot t$ is a constant. This indicates that the Schütz-Borissov rule applies to the experimental data, and accordingly, it appears that asclepain is inhibited by combination with the digestion products of hemoglobin.

It is interesting to recall that in the case of milk clotting, where the same degree of change (the clotting point) is measured for different enzyme concentrations, the product of enzyme concentration and clotting time is likewise a constant. It is possible that the product of milk clotting action may cause the inhibiting effect observed by Balls and Hoover, and that milk coagulation is an example of the Schütz-Borissov law.

SUMMARY

1. The kinetics of milk clotting by asclepain, the protease of *Asclepias speciosa*, were investigated. At higher concentrations of enzyme, the clotting time was inversely proportional to the enzyme concentration.

2. The digestion of casein and hemoglobin in 6.6 M urea by asclepain follows the second order reaction rate. The rate was roughly second order for casein in water.

3. Evaluation of the nature of the enzyme-substrate intermediate indicates that one molecule of asclepain combines with one molecule of casein or hemoglobin in urea solution.

4. Inhibition by the reaction products was deduced from the fact that the digestion velocity of hemoglobin in urea solution varied with the asclepain concentration in agreement with the Schütz-Borissov rule.

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PHYSICOCHEMICAL PROPERTIES OF THE PROTEOLYTIC ENZYME FROM THE LATEX OF THE MILKWEED, ASCLEPIAS SPECIOSA TORR. SOME COMPARISONS WITH OTHER PROTEASES

III. Kinetics of the Heat Inactivation of Papain, Bromelin, and Asclepain

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INTRODUCTION

The study of heat inactivation is often of considerable value in the characterization of proteolytic enzymes. This is true because in most cases these proteases appear to be inactivated at different rates at the same temperature and pH values. Also the determination of the inactivation velocity constants at different temperatures makes it possible to evaluate the critical thermal increment of the enzyme.

The thermal inactivation of crystalline trypsin, chymotrypsin, and pepsinogen is completely reversible (1), so that the denatured inactive enzymes formed by heating the solutions revert to the native condition on cooling. In the present study of the thermal characteristics of three plant proteases, papain, bromelin, and asclepain,[1] the heat inactivation could not be reversed by cooling the solutions. The rates of destruction of these enzymes do not show the great dependence on pH which pepsin exhibits (2). Near neutral pH, at constant temperatures, these plant proteases are inactivated at rates which can be described in most cases by simple equations. Differences in the state of purity do not seem sufficient to account for the individual behaviors of the enzymes.

Experimental Procedures

Enzyme Solutions. Papain.—Merck's papain powder was partially purified by first salting out the enzyme (from an aqueous extract buffered at pH 7) with $(NH_4)_2SO_4$

[1] The protease of the milkweed, *Asclepias speciosa*, whose preparation and properties have been described in the two previous papers of this series (Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, 1940, **23**, 275, 289).

added to about half saturation, and then precipitating the redissolved enzyme with two volumes of 95 per cent alcohol. The product, washed with 70 per cent alcohol and dried in a vacuum desiccator, was more than twice as active as the original material. The solution used for the inactivation studies contained 0.33 mg. papain per ml. It was activated with dilute NaCN and then adjusted with KH_2PO_4 to pH 7.0.

Bromelin.—This enzyme was prepared from fresh pineapple fruit by the method of Willstätter, Grassmann, and Ambros (3). A solution was used which contained 1.5 mg. enzyme, per ml., activated in the same manner as papain.

Asclepain.—The solution contained 2 mg. of this enzyme per ml. of pure water. It was not buffered, and its exact pH was not known.

Method for Measuring Rates of Inactivation.—A series of small test tubes, each containing about 1.5 ml. of a given enzyme solution, were immersed in a thermostat at the desired temperature (±0.1°). The tubes were gently shaken for about a half minute and then corked, so that no water could evaporate. After varying times of heating, each tube was plunged into an ice water bath, which quickly stopped the destruction of enzyme.[2] The corked tubes were tilted and rotated horizontally to collect condensed moisture on the wall. Then the residual proteolytic activity of each solution, as well as that of the unheated enzyme solution, was measured in 1 ml. aliquots by Anson's hemoglobin method (4). The substrate, containing 2 per cent hemoglobin in about 6.6 M urea, buffered at pH 7.0, was digested for 15 minutes at a temperature of 30°.[3] The proteolysis is expressed in terms of the color value of tyrosine produced in 6 ml. of digestion mixture.

Experimental Results and Interpretation of Reaction Rates

Papain.—The rate of thermal inactivation of papain at 75, 80, and 83° was found to follow the equation of a simple first order reaction

$$2.3 \log \frac{A_0}{A} = Kt$$

where A_0 is the activity of the unheated enzyme solution, A the residual activity after heating for the time, t, and K the velocity constant. This is shown in Fig. 1. By plotting the experimental values of log A against t, straight lines were obtained, in agreement with the requirements of the first order equation.

By taking log A_0 as the intercept on the ordinate, K can be evaluated for each temperature. The constants for the different temperatures can also be calculated by substituting the experimental values of A_0, A, and t directly into the first order equation. The curves in Fig. 1 seem to depart very slightly from linearity toward the end portions, which correspond to

[2] Only a few seconds are required for the heating and cooling of these small volumes, and the time lags in starting and stopping the inactivation largely cancel each other. The time of heating was measured with a stop-watch.

[3] There is no appreciable destruction of enzyme at this temperature.

the greatest degree of inactivation. When Merck's papain was used without preliminary purification, the order of the inactivation was indeterminate.

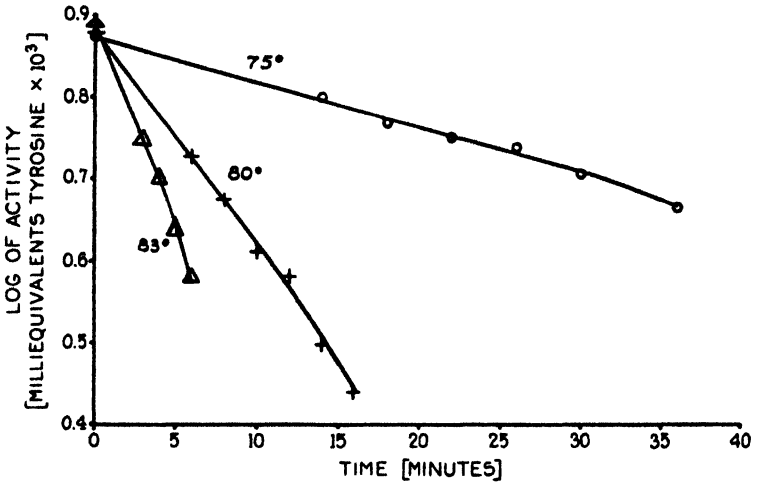

FIG. 1. Rate of heat inactivation of papain

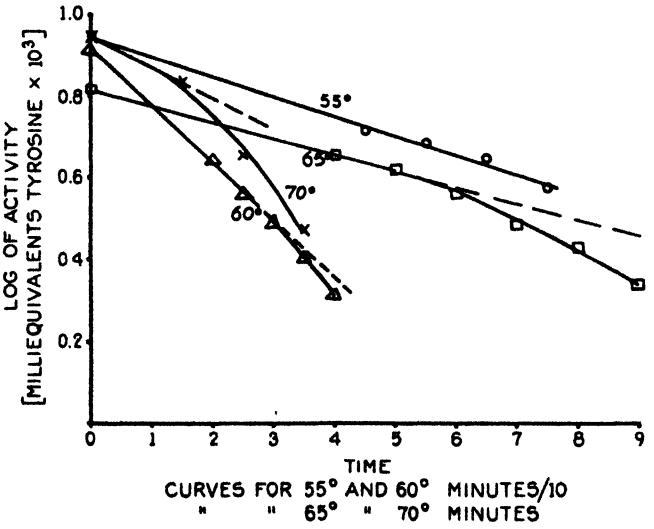

FIG. 2. Rate of heat inactivation of bromelin

Bromelin.—The rate of inactivation of this protease was found to follow the first order equation at 55°, and nearly so at 60°, but at higher temperatures the destruction of enzyme was greater than the first order equation

required. This was shown by the gradual rise in the values of the velocity constants, which were calculated from the first order equation.

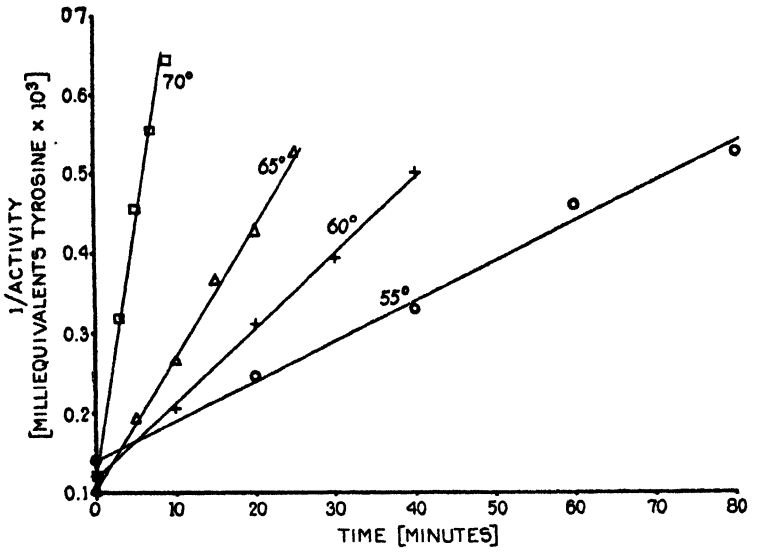

FIG. 3. Rate of heat inactivation of *Asclepias* protease

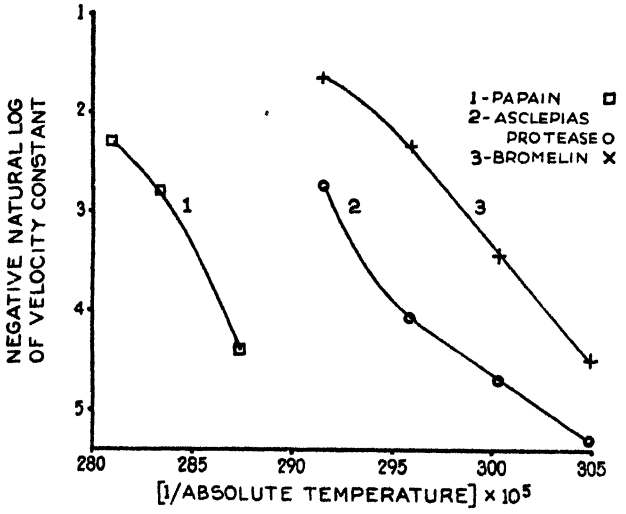

FIG. 4. Relation between velocity constants and absolute temperature

In the plots of log A against t, given in Fig. 2, the deviations from linearity are more marked than in the case of papain, and increase progressively with increase in temperature. It was necessary to use the initial linear

portions of the curves in order to evaluate graphically the velocity constants, particularly at 65 and 70°.

It was thought possible that the deviations from the first order rate were due to the inhibition of part of the active enzyme by combination of the latter with the inactive fraction. If this hypothesis were correct, one would expect a mixture of active (unheated) and completely inactivated enzyme to have less activity than a solution having the same concentration of the active enzyme alone. This test was performed, and it was found that the former solution did have slightly less activity than the solution of only the active enzyme. But this difference was too small to account for the observed deviations in the inactivation rates. It may be noted that Michaelis and Rothstein (5), in their study of the inactivation of rennet by alkali, found that the inactive enzyme did not influence the rate of destruction of the remaining active portion.

Asclepain.—This enzyme differed qualitatively from papain and bromelin in that its inactivation followed the course of a second order reaction at 55, 60, 65, and 70°. The second order equation may be written as

$$\frac{1}{A} - \frac{1}{A_0} = Kt.$$

The letters have the same significance as before.

In Fig. 3 it is seen that the plots of $1/A$ against t give straight lines in accordance with the second order equation. K was evaluated from the slopes of the lines according to the relation $\frac{d(1/A)}{dt} = K$. If the experimental data are substituted into the second order equation, it is found that the velocity constants for each temperature do not vary beyond the limits of experimental error. The data do not fit the first order equation.

Correlation of Velocity Constants

The heat inactivation of pure enzymes, such as crystalline pepsin (6), almost invariably follows the first order equation. It is interesting that the inactivation of impure papain and bromelin also follows this reaction order (at certain temperatures). The only hitherto recorded instance of second order inactivation of an enzyme is that reported by Kunitz and Northrop (7) for crystalline trypsin. Between pH 2.0 and about 9.0 the irreversible inactivation of this protease is a second order reaction. The explanation offered is that the active native protein (trypsin) hydrolyzes the denatured form with which it is in equilibrium. The investigation of the second order inactivation mechanism in the case of asclepain was not feasible, due to the impure condition of the enzyme preparation.

The average values of the velocity constants of papain, bromelin, and asclepain are recorded in Table I. Since the first and second order constants are not strictly comparable, it is more convenient to use the half life period[4] to compare the rates of inactivation of the three enzymes. Using this criterion, it is seen that the rate of destruction of papain at 75° is about equal to that of bromelin at 55° and only about half as great as the rate for asclepain at 55°. At 70° bromelin and asclepain are inactivated more than 20 times as fast as is papain at 75°. It is clear that papain is by far the most resistant to heat inactivation, and that the thermal

TABLE I

Comparison of Thermal Characteristics of Three Plant Proteases

Enzyme	Order of reaction for heat inactivation	Temperature	Velocity constant (from curves)	Velocity constant (from equations)	Half life period (from curves)	Critical thermal increment
		degrees	*K × 100*	*K × 100*	*min.*	*cals. per mol*
Papain	First	75	1.23	1.26	56	
		80	6.0	6.1	11.5	75,000 (75–80°)
		83	11.0	11.2	6.3	51,000 (80–83°)
Bromelin	First	55	1.12	1.11	62	
		60	3.2	3.3	21.5	46,000 (55–60°)
		65	8.8	9.3*	7.9	45,000 (60–65°)
		70	19.2	—	2.6	36,000 (65–70°)
Asclepain	Second	55	0.51	0.50	28	
		60	0.95	0.92	13	27,000 (55–60°)
		65	1.65	1.65	6.5	25,000 (60–65°)
		70	6.4	6.3	1.9	61,000 (65–70°)

* Average of values for the 4, 5, and 6 minute preheating times.

stabilities of bromelin and asclepain (particularly between 65–70°) are not very different.

The temperature coefficients of the destruction rates are best considered in their relation to the corresponding energies of inactivation, the critical thermal increments. These latter values were calculated for the three enzymes with the aid of the van't Hoff-Arrhenius equation

$$\frac{d \ln K}{dT} = \frac{E}{RT^2}$$

which relates the reaction velocity constant, K, the absolute temperature, T, and the critical thermal increment, E. R is the gas constant.

[4] The time required for the proteolytic activity to be reduced to half its initial value.

Integrated, this equation may be written in the form

$$\ln K = -\frac{E}{1.98T} + C.$$

According to this equation, the plot of ln K against $1/T$ should give a straight line whose slope is $-E/1.98$. The plot of these variables, given in Fig. 4, shows some departure from straight lines, particularly for the curves of bromelin and asclepain at higher temperature values. This suggests that E is not constant for the whole of the temperature ranges. If the regions of 75–80° for papain, and 55–65° for bromelin and asclepain, are considered as linear, the critical increments (in calories per mol) as calculated from the slopes are: papain, 75,000; bromelin, 48,000; asclepain, 27,000.

The van't Hoff-Arrhenius equation integrated between the limits T_2 and T_1 is

$$\ln \frac{K_2}{K_1} = \frac{E(T_2 - T_1)}{1.98\,T_1T_2}.$$

The equation in this form was used to calculate E for the separate temperature intervals. The resulting values of the critical increments given in Table I are seen to correspond to those evaluated from the curves.

The critical increments for papain and bromelin are apparently of the same high order as the values which are reported in the literature for several other enzymes and substances closely related to enzymes. Asclepain (55–65°) has a somewhat lower value of E.

Steinhardt (2) and La Mer (8) have concluded that the values for the energy and entropy of enzyme denaturation and for protein denaturation generally are illusory, since the comparisons of rates at constant pH alone may be fallacious. In the case of pepsin, the customary method measures in addition to the energy of activation, the heat of dissociation of groups involved in acidic equilibria. When this factor was allowed for, the true critical increment becomes 18,300 instead of 63,500 calories for this enzyme. In the present study of inactivation, the effects of varying pH on the rate of destruction were not studied, but it seems possible that the differences in critical increments for the plant proteases are in part due to different heats of dissociation of groups in the enzyme molecules. In any event, the high critical increments, particularly those of papain and bromelin, suggest that heat inactivation involves the breaking of a number of bonds in the enzyme molecule, as is the case in protein denaturation. This agrees with the evidence for the protein nature of plant proteases.

SUMMARY

1. The rates of heat inactivation of papain, bromelin, and asclepain were determined at several different temperatures. Papain was by far the most resistant to heat.

2. The destruction of papain at 75–83° and bromelin at 55–70° followed the course of a first order reaction, except that for longer times of heating, bromelin (at 60–70°) was inactivated more rapidly than the first order equation required.

3. The rate of inactivation of asclepain at 55–70° followed the second order equation.

4. The critical thermal increments of inactivation of papain and bromelin, calculated with the van't Hoff-Arrhenius equation, were of the same high order that has been found for protein denaturation. The increment for asclepain was somewhat lower.

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ON THE RELATION BETWEEN BIRTH WEIGHT AND LITTER SIZE, IN MICE

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I

With multiparous mammals a particular relationship exists between the mean weight (W) of litters of young of number N and the number in the litter (Enzmann and Crozier, 1934–35; Crozier and Enzmann, 1935–36). The data on various animals obey sufficiently well the equation

$$W = aN^K, \tag{1}$$

so that on a double log grid the values of W and N adhere to a straight line with slope $= K$; $a =$ the ideal weight of a litter of 1 individual. The constant K is nonspecific.

The use of equation (1) demands homogeneous data. Its applicability is easily obscured by genetic heterogeneity of the assemblage of breeding animals, and by such factors as litter order. Thus, if the latter is not controlled in an otherwise homogeneous lot, larger litters will tend to be borne by older, heavier animals which have already carried young, so that in a large (and in this respect sufficiently non-homogeneous) series equation (1) may still be followed but with a larger value of the exponent K. An illustrative case is given in Fig. 1.

In reasonably homogeneous series K is apparently constant with the value 0.83, as obtained with different races of mice, rats, rabbits, pigs. Its nonspecific invariance has been interpreted as due to phenomena consequent upon the partitioning of nourishment among the developing young in a litter. Thus it is sufficient to consider that each increment of 1 in a litter is responsible for a constant fractional increase in the material supplied to the young by the mother, and that (on the average) this material is evenly partitioned among the young. This should be most simple to test in a mammal giving no relationship of litter size to period of gestation. It is nicely confirmed by the changes in weight during the nursing period, as related to the time course of milk supply by the mother, in litters of various sizes (Crozier and Enzmann, 1935–36). The interpretation of K

as an index of partition thus suggests that so long as the genetic nature of mother and young is substantially the same, the constant K should be nonspecific.

It was pointed out, however (Crozier and Enzmann, 1935–36), that if litters were to be formed containing several kinds of young differing in capacity for growth, then this conception of the rôle of the partitioning of

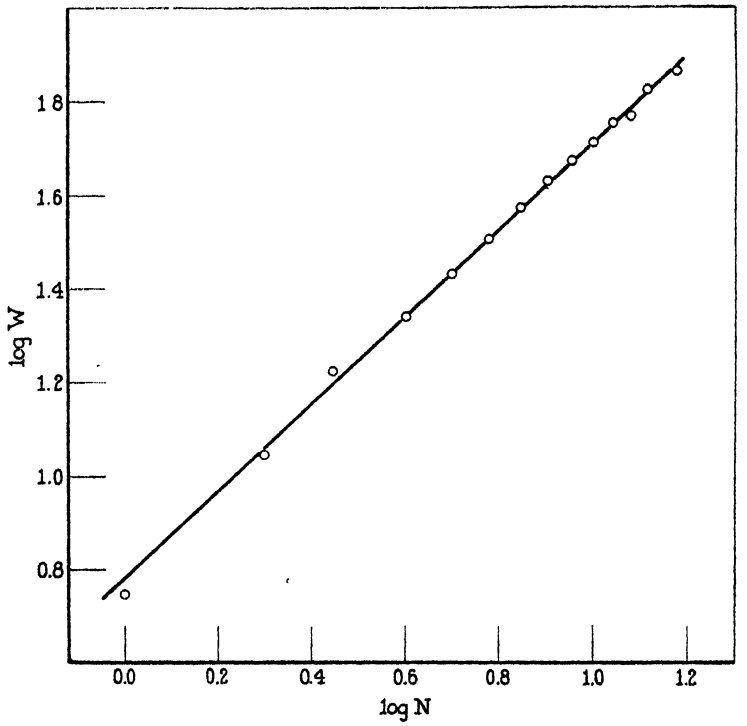

FIG. 1. Mean litter weights W, as a function of number N in the litter, gray Norway rat from data of King (1935); 6,295 unsuckled young in generations 8 to 26, mothers of various ages. The line gives an exponent = 0.93; see text.

nutriment could be tested experimentally. Its importance for certain aspects of the theory of growth made it desirable to undertake such a test. The nature of equation (1) and of the constants a and K brings it about that it is also possible to make several predictions with reference to genetic studies upon birth weights (Crozier and Enzmann, 1935–36), which can in part be tested.

Equation (1) is of the form utilized by Robb (1929), Huxley (1932), Teissier (1934), and others in their studies of relative growth. It has been employed among other things

for the correlation of the linear dimensions and of the weights of parts. It is doubtful if the theory underlying its use really makes these varied applications properly congruous (*cf.*, *e.g.*, reviews in Brody, Davis, and Ragsdale, 1937; Glaser, 1938). For data on the relative sizes or weights of parts of an organism at different times, Huxley and Teissier (1936) have suggested that the equation for relative growth (allometry) be used with an agreed-upon uniformity of symbols, $y = bx^a$, to avoid confusion. Since we are here dealing with a distinctly different sort of situation, involving *weight* after a fixed growth time as a function of *number* in a litter, it seems desirable to retain the terminology used in the earlier papers.

It is possible to show that for situations of this kind the common occurrence of power function relationships with fractional exponents may be expected on purely dimensional grounds (Crozier and Holway, 1939–40).[1] This of course tells nothing about the mechanism whereby they arise in particular instances. In any case, an experimental test of the physical properties of the exponent K is required before the equation can be regarded as used in a significant analytical way.

II

To establish litters containing two sorts of young having distinct growth potentials we have proceeded by crossing our inbred *albino* mice to an *anemic* (*dwarf*) strain. By inbreeding F_1's, and by backcrossing, it was hoped to obtain litters of different numbers and in each number-class a diversity of proportions of dwarf to normal young. For various reasons it was not practicable to complete the somewhat extensive program originally contemplated. The data do, however, test the main points already outlined.

The *albino* stock (AA) was the same as that which we have used previously (Crozier and Enzmann, 1935–36). The *anemic* stock (aa) (*cf.* Mixter and Hunt, 1933) is one characterized by having the young temporarily defective in hemoglobin and in erythrocytes at birth, and weighing distinctly less than do the young of ordinary mice; they are lighter in color (bluish pink). The defect is less apparent after age 10 days, may disappear at about 2 weeks, and the adults are of ordinary size and weight. The anemic defect is probably due to a recessive. We found considerable difficulty in maintaining a pure aa stock. I am indebted to Dr. G. Pincus, Mr. J. Berkman, and Miss Sheila Dehn (Mrs. M. P. Gilmore) for assistance in the course of the experiments.

The relation between mean litter weight and number in litter is obtained from Table I for aa (anemic), and F_1 (Aa), and for AA (non-anemic) from the preceding paper (Crozier and Enzmann, 1935–36). As Fig. 2 shows, the data are describable in each case as a parabola, $W = aN^K$ in which K is sensibly the same while a differs. For aa and F_1 mice the value of K is

[1] Pages 116 *et seq.*

that already obtained (Crozier and Enzmann, 1935–36) for various mammals, 0.83. The value of a or W_1 (the ideal weight of a litter of 1), is characteristic of the stock; for F_1 it is strictly intermediate between that for AA and for aa:

AA	W_1	= 1.76 *gm.*
F_1		= 1.67
aa		= 1.52

It is to be noted that if birth weights were to be compared simply on the basis of observed mean individual weight at birth, without regard to litter

TABLE I

Weights of litters at birth, for first litters with various numbers of young, in an anemic strain (aa) and for F_1 (Aa) resulting from matings of anemic males with albino (AA) mothers. Numbers of litters given in parentheses. S.D. of mean W is 0.1 to 0.3 gm. σ_1 = 0.04 to 0.16 gm. for the individual weights. See Fig. 2.

N	W_m, *gm.*	
	Anemic (aa)	F_1 (Aa)
2	—	2.86 (1)
3	3.81 (1)	4.28 (2)
4	4.96 (4)	5.33 (3)
5	6.00 (8)	6.45 (6)
6	6.73 (22)	7.42 (19)
7	7.99 (7)	8.64 (21)
8	8.08 (3)	9.54 (29)
9	9.28 (3)	10.72 (25)
10	—	11.83 (9)
11	—	12.14 (4)
12	—	13.45 (3)
13	—	14.01 (1)
14	—	15.94 (1)

size, a different state of affairs could be claimed, suggesting "heterosis." This is due to the fact that the mean litter number and σ for litter number are not the same for the three groups of mice (*cf.* Fig. 3):

AA—litter number *median*	= 6.5 ± 1.5
F_1	= 7.4 ± 1.2
aa	= 5.3 ± 0.8

For aa the median litter number is smallest, and the spread (P.E.$_1$) is less. Median N is greatest for F_1, and its scatter is intermediate (Fig. 3). Thus the median litter size does exhibit "heterosis," although the variability and the ideal weight of a litter of 1 (*i.e.*, freed from the effects of growth

competition) are strictly intermediate between these properties of the two parent stocks. These facts lead to the expectation that the analysis of the data on compound litters (Section III) may safely proceed in terms of number and genetic type (*Aa* or *aa*).

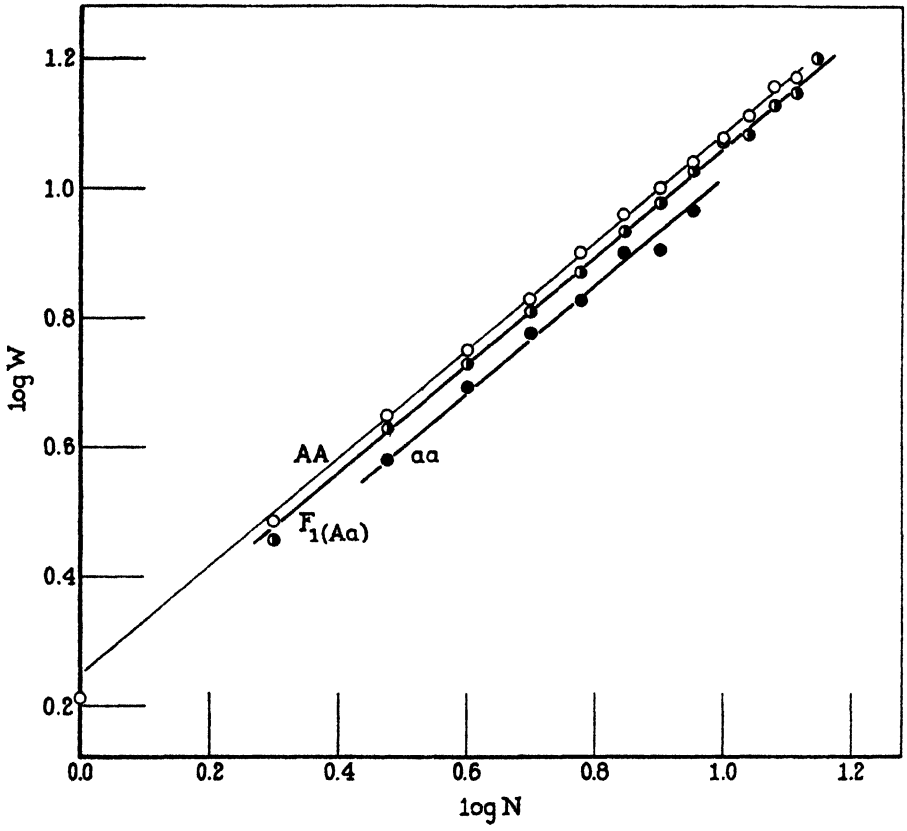

FIG. 2. The relation between W and N for albino (AA) and non-albino anemic (aa) mice, and for their F_1 offspring, can be effectively described as parabolic, with the same exponent (0.83). Data in Table I. See text (AA data from Crozier and Enzmann, 1935–36).

The *Aa* litters and the *AA* are both carried by *AA* mothers. The fact that intrauterine growth depends upon an interaction between mother and young makes it probable that the development of litters carried in *Aa* mothers should be influenced by the properties of the mothers. To test this we may examine data on backcross litters (*Aa* ♀ × *aa* ♂) containing *no aa* young (Table II). The numbers are not large (76 litters in the first, 81 in the second). They show, however, that *Aa* young in *Aa* mothers

are, litter for litter, heavier than *Aa* young in *AA* mothers in litters of 6 or more. This may, of course, be due to the fact that the F_1 mothers (*Aa*) are heavier than the *AA*. The slope of the log plot of *W vs. N* for these all-*Aa* litters (Fig. 4) is definitely higher than for the F_1 litters (Fig. 2). This could be a consequence of intrauterine elimination of some *aa* young. In that case W_1 would be higher for the backcross *Aa*'s, about the same as for *AA* young in *AA* mothers, and less than for the *Aa*'s. Comparison

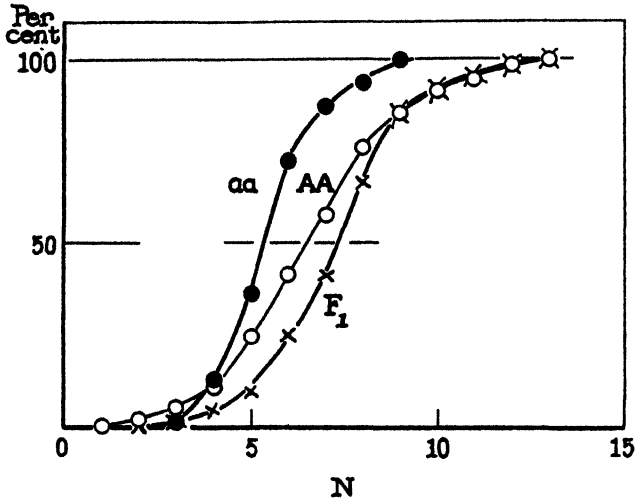

FIG. 3. Summed frequency curves for observed occurrence of litters of *N* in stocks of *aa*, *AA*, and F_1 (*aa* ♂ × *AA* ♀).

TABLE II

Weights of litters (*W*) from *aa* ♂ × *Aa* ♀, for litters containing no anemic young at birth, all individuals being apparently *Aa*. Number in litter = *N*.

N	3	4	5	6	7	8	9	10	11
W, *gm*. .	3.84(6)	5.42(4)	6.38(10)	7.71(27)	8.85(19)	10.10(15)	10.98(14)	12.60(2)	13.44(1)

of Tables I and II shows that for litters of 6 or more at birth the backcross litters with no *aa* individuals consistently weigh a little more than do the F_1 litters of the same *N*; below *N* = 6 the values of *W* do not differ significantly. This suggests that for the larger backcross litters there has been a significant elimination of *aa* young during early development.

III

In the backcross litters we have varying numbers, and for each *N* a variety of proportions of *aa* to *Aa*. The data are of course possibly com-

plicated by the influence of "growth-influencing genes," as well as by the selective elimination of young. It could easily be considered that the anemic young should be at a disadvantage. There is on the whole a slight

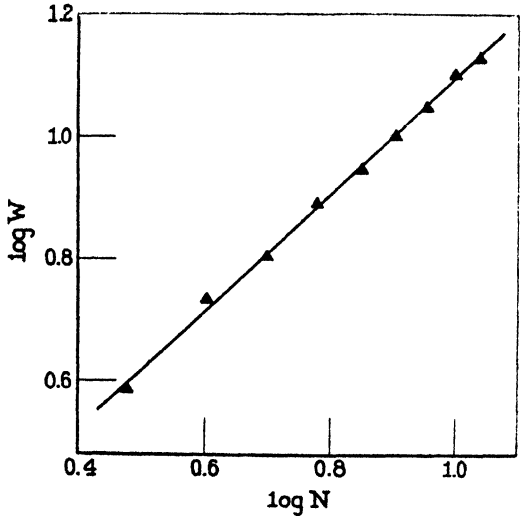

FIG. 4. Relation between number in litter (N) and litter weight (W) for backcross litters ($aa \times Aa$) containing no anemic young at birth. The slope gives $K = 0.95$, higher than for homogeneous F_1 litters; see text.

TABLE III

Mean weights, *gm.*, of *aa* individuals and of *Aa* individuals in backcross litters of different sizes (N), when the *aa* and *Aa* are of equal numbers in the litter. (For N odd, correction has been used as described in the text.) See Fig. 5.

N	W,*aa*	W,*Aa*
2	0.72	1.20
4	2.08	2.68
5	2.56	2.98
6	3.63	4.38
7	3.90	4.43
8	4.48	5.13
9	4.73	4.93
12	6.34	6.66

deficiency in anemic young. Of 1,140 backcross young, 223 were recognized as anemics,—a ratio of 1:4.11 rather than 1:3.0. The effect should be more apparent with larger litters, since in that case competition with faster growing non-anemic littermates should be more severe. The data show that both in the backcross generation and in F_2 the proportion of

litters exhibiting some *aa* young increases steadily with the size of the litter, as it would be expected to do on a basis of chance; however, the ratio of *aa*/*Aa* is not independent of litter size but falls as N increases, both in backcross litters and in F_2.

We desire to know how the presence of *Aa* and *aa* young together in the same litter affects the weights of each at birth. The assumptions connected with the use of the parabolic relation of W to N call for a proportionately constant increase of nutriment from the mother for each increase of 1 in the litter, the partitioning of this material between mother and young, and among the young. The simplest conditions for a test are thus given by litters in which there are equal numbers of anemics and of non-anemics (Table III). Each group may be taken as one growing mass, with the same number of growth centers in each case. (For litters with N odd, correction has been made by adding to the weight of one group 0.5 times the *mean* weight for its type and number, and subtracting the corresponding quantity for the other group. The slope of the line in Fig. 5 is not affected if these cases are omitted.) We therefore expect that the division of nutritive material will take place in such a way as to obey the partition rule. Fig. 5 shows that this is the case. For the various litter sizes $\log W_A = 0.83 \log W_a + const.$, where the subscripts A and a refer to the phenotypes. The slope constant 0.83 as drawn is identical with that for the relation between W and N in each of the pure types (section II). It follows that the ratio of the rates of increase in weight per unit increase of number in litter is directly proportional to the ratio of the weights:

$$dW_A/dW_a = 0.83\, W_A/W_a.$$

On general grounds it is reasonable to suppose that the supply of nutriment by the mother is a function of the synthetic activity of the young, as well as of their number. The analogy with milk secretion (Crozier and Enzmann, 1935–36) is confirmatory. Consequently we should expect that with mixed litters, 50 per cent type *a*, the anemic half should tend to weigh more at birth than half the weight of a pure anemic litter of the same number. This is the case for $N > 5$ (*cf.* Tables I and III). The interrelations of weights of anemic and non-anemic young in litters of given N when the number of anemics increases, as discussed later, show that this relationship cannot be entirely, or perhaps not even primarily, due to the fact that the F_1 mothers are a little heavier than either *AA* or *aa* mothers. The relation is to a fair approximation parabolic, the increase of W_a per unit increase in N being greater the larger the number in the litter (Fig. 6), and in proportion to the ratio of the weights. Similarly, as N increases the weight of the 50 per cent non-anemics in these mixed litters increases a

little faster than the weight of one half normal non-anemic litters of the same numbers, and than (Fig. 7) the weight of one half non-anemic back-cross litters (also in *Aa* mothers), the ratio of the rates of increase being again directly proportional, nearly enough, to the respective weights.

The interpretation of such a relationship on grounds other than of partitioning of nutriment in proportion to number and "drawing power" of the

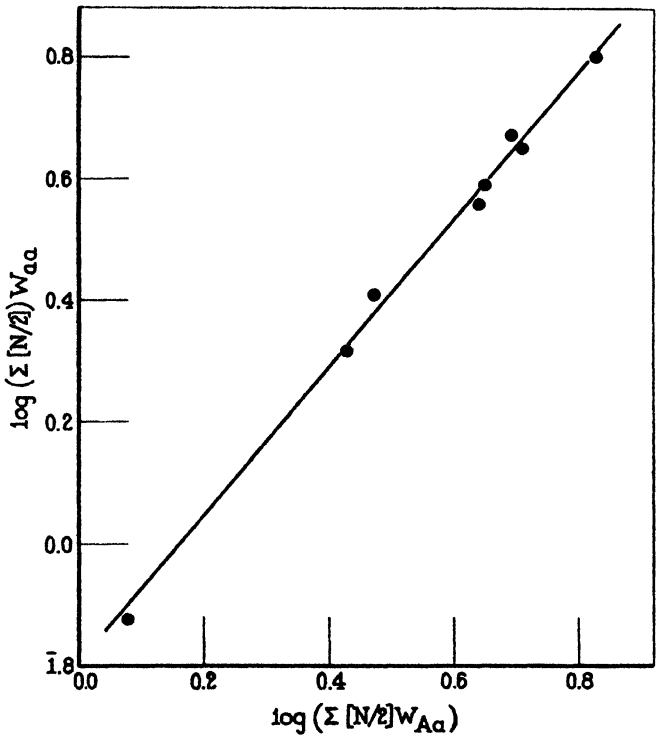

FIG. 5. Relation between weights of *aa* and of *Aa* individuals in litters of various sizes (N), when *aa* young constitute $N/2$ of the individuals; see text; the slope (0.83) is the same as in Fig. 2.

developing young appears difficult. On the other hand it is easily accounted for by that conception. With it additional facts are fully consistent. Litters of 6, 7, and 8 are sufficiently numerous in our data to permit a test of the effect of increasing proportion of anemics to non-anemics. As N increases, more nourishment is provided by the mother; the call for foodstuffs is greater with the non-anemic young, and the provisioning is greater; but as the proportion of non-anemics is found greater the anemic young should get less and less of it, on the partition view. We find that the mean weight of 1 anemic at birth in a litter containing 6, 7, or

8 young (the remainder being non-anemic) is: 1.25 gm., 1.09 gm., 1.21 gm. respectively, distinctly above the corresponding mean weights for 1 anemic in pure *aa* litters in *aa* mothers (1.12, 1.14, 1.01 gm.). The ratio of the

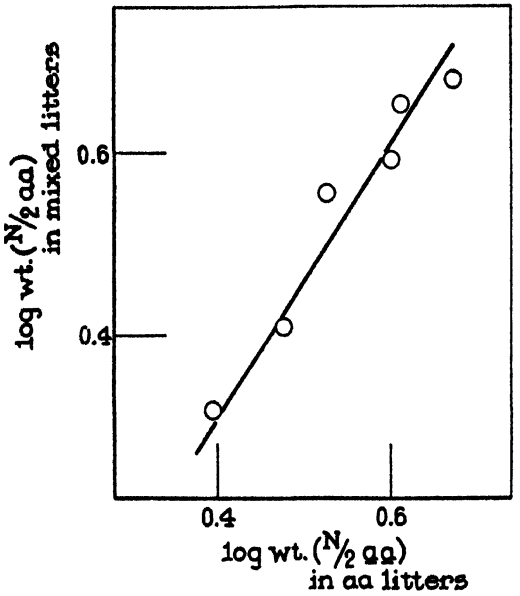

FIG. 6

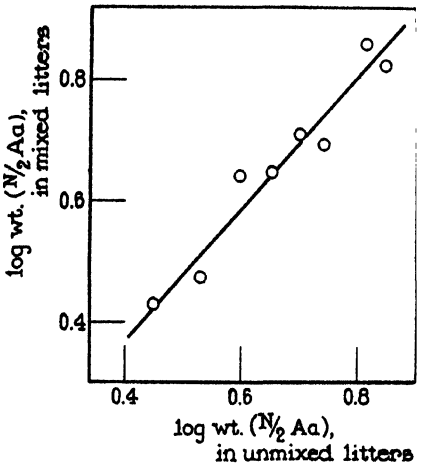

FIG. 7

FIG. 6. Relation between weights of *aa* individuals (constituting half the litter) in mixed and in pure *aa* litters; a given number of *aa* young, in the presence of the same number of *Aa*, tend to be heavier at birth than if (in an *aa* mother) they are accompanied by the same number of *aa*; the rate of increase in W per unit increase of N is greater for *aa* in the mixed litters.

FIG. 7. The weights of a given number of *Aa* (non-anemic) young, constituting half a litter (in *Aa* mothers) of which the other half is *aa*, are less, on the whole, than those of the same number of *Aa* young in pure (F_1) litters, but, number for number, these weights increase faster with N than if the litters contain no *aa* individuals. This is tested by plotting log W_{Aa} for the non-anemic halves of mixed litters against log W_{Aa} for one half the litter when no anemic young appear, in the same backcross.

mean birth weight of a non-anemic in a mixed litter to that of an anemic increases as the proportion of anemics increases, with N constant:

	$(Aa_1)/(aa_1)$ for increasing numbers of aa:				
	(1)	(2)	(3)	(4)	(5)
$N = 6$	1.09	1.07	1.21	1.13	
$N = 7$		1.10	1.13	1.16	1.16
$N = 8$	1.02	1.17	1.08	1.14	1.18

So long as there are non-anemics in the litter, the mean weight of the anemics is above that of the same number in a litter (of the same N) of pure anemics. This is due to the greater amount of material provided by the mother as a consequence of the presence of the faster-growing non-anemics, which is shared in by the *aa* young. On the other hand, the mean weights of non-anemics in the mixed litters are also greater than in pure litters (F_1) of *Aa*, since a larger proportion of the material is obtained by them, as already demonstrated. In litters of 6, 7, and 8 the mean weight of 1 non-anemic ranges from 1.22 to 1.46 gm.; in F_1 *Aa* litters, from 1.19 to 1.24 gm.

TABLE IV

Mean weight of 1 anemic (aa_1) in litters containing 1 or 2 anemics together with 2 to 11 non-anemics.

Non-anemics	aa_1, gm.	
	1 *aa*	2 *aa*
2	0.72	
4	0.90	1.17
5	1.38	1.25
6	1.25	1.12
7	1.10	1.09
8	1.19	1.14
9		1.12
10	1.14	1.15
11	0.89	1.12

This situation may be taken to provide a kind of model for the conception of heterosis as due to disharmony in development (*cf.* Crozier and Pincus, 1931 32). A *mixed* litter of anemics and non-anemics gives mean birth weights for each type which are on the whole *above* those encountered in unmixed litters, as an automatic consequence of the partitioning of food materials between mother and young.

There must be limits beyond which this effect is modified. We have tabulated (Table IV) the mean weights of 1 and 2 anemics in mixed litters containing various numbers of non-anemics. The number of available cases is not large; however, the weight of 1 anemic does appear to go through a maximum as N increases, as the foregoing analysis requires.

SUMMARY

For mice, as for various other mammals, the relation between number N of young in a litter and the weight W of the litter can be expressed as

$W = aN^K$. For adequately homogeneous data K has the nonspecific value 0.83. With data not homogeneous with respect to certain conditions the equation may still be descriptive, but with K higher than 0.83.

Two kinds of mice obeying this formulation, with the same K, are an albino strain (AA) and a flex-tail foetal anemic (aa). Their ideal weights of a litter of 1 (W_1, free from effects of intrauterine competition) are quite different. Their F_1 offspring (from AA mothers) give W_1 precisely intermediate.

To test the partition theory for the basis of the parabolic equation, back-cross and F_2 litters were obtained in which for a span of litter sizes there occurred various proportions of anemic to non-anemic young. For equal numbers of each in the same litters the relation of weight of aa to weight of Aa young is again described by $W_a = aW_A^K$, and as before $K = 0.83$.

Examination of the weights of anemic and of non-anemic young, for various proportions of the two in litters of different total numbers, shows that the partition theory can account for a number of the curious relations, including the fact that aa young and Aa young if in mixed litters increase in weight more for an increment of 1 in the litter than if in unmixed litters of the same N. This mechanical result of partitioning can be regarded as a kind of model for heterosis resulting from developmental disharmony.

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THE REACTIONS OF IODINE AND IODOACETAMIDE WITH NATIVE EGG ALBUMIN*

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INTRODUCTION

The hydrolysate of egg albumin contains cysteine (Mirsky and Anson, 1935). Denatured but unhydrolyzed egg albumin gives the nitroprusside test for SH (Hefter, 1907) and reduces the weak oxidizing agents glutathione and cystine, which presumably can oxidize only SH groups (Hopkins, 1925; Mirsky and Anson, 1935).

No SH groups have hitherto been detected in native egg albumin. Native egg albumin does not give the nitroprusside test (Hefter, 1907). It does not reduce ferricyanide even at pH 9.6 (Mirsky and Anson, 1936 *b*). It does not reduce the still stronger oxidizing agent, porphyrindin at pH 7.0 (Greenstein, 1938).

Similarly S–S groups (Walker, 1925) and tyrosine and tryptophane groups (Mirsky and Anson, 1936) which are detectable in denatured egg albumin are not detectable by the same tests in native egg albumin. The problem of why various groups appear in detectable form when egg albumin is denatured is one of the central problems of the theory of protein structure.

The present experiments show that native egg albumin can react with iodine and iodoacetamide despite the fact that it does not react with ferricyanide and porphyrindin. They suggest further that the groups which react with iodine and iodoacetamide are either SH groups or are at least the groups which become SH groups when the protein is denatured.

Quite apart from the theory of the iodine and iodoacetamide reactions it is of practical importance for experiments with enzymes and virus proteins to know that a protein of the egg albumin type which contains cysteine but which gives a nitroprusside test only when denatured can be modified by reactions of the native form of the protein with iodine and iodoacetamide.

* A brief account of the reactions of iodine and iodoacetamide with native egg albumin has been published in *Science* (Anson, 1939 *a*).

If the iodine reaction is carried out in acid solution, furthermore, this modification of the native protein can be carried out without any reaction of iodine with tyrosine groups.

The reactions between native egg albumin and iodine and iodoacetamide which will be described provide new facts which must be explained by any theory of the structure of native egg albumin and of the reason for the appearance of the nitroprusside test when the protein is denatured.

TABLE I

Amount of Ferricyanide Reduced by 10 Mg. Denatured Egg Albumin in Duponol PC Solution after Reaction of Egg Albumin in Native Form with Iodine at 37°C. for 5 Minutes

pH at which iodine is added	Iodine added	Ferrocyanide formed
	milliequivalents	*milliequivalents*
6.8	0.0	0.001
6.8	0.001	0.00046
6.8	0.002	0.00024
6.8	0.004	0.00010
4.7	0.001	0.00027
4.7	0.002	0.0001
3.2	0.001	0.00017
3.2	0.0015	0.0001

The Reactions of Iodine and Iodoacetamide with Native Egg Albumin

10 mg. of denatured egg albumin in a neutral solution containing the detergent Duponol PC reduces 0.001 mM of ferricyanide. This reduction of ferricyanide is due in part at least to SH groups (Anson, 1939*b*).

If native egg albumin is treated with iodine and then denatured, the denatured protein does not give the nitroprusside test and does not reduce dilute ferricyanide in neutral Duponol solution. 1 cc. of 0.0015 N iodine is required to abolish the ferricyanide reaction with denatured egg albumin, if the reaction between iodine and 10 mg. of native egg albumin is carried out at pH 3.2. If the reaction is carried out at pH 6.8, 1 cc. of 0.004 N iodine is required (see Table I). In both cases all the iodine added is used up in the reaction with native egg albumin.

If native egg albumin is treated with iodoacetamide at pH 9.0 and then denatured, the denatured protein reduces only 60 per cent as much ferricyanide as is reduced by denatured egg albumin which has not been treated with iodoacetamide.

The Reactions of Iodine and Iodoacetamide with Amino Acids and Cysteine-Free Proteins

Before discussing the interpretation of the reactions of native egg albumin with iodine and iodoacetamide I shall state what is known from previous experiments and new experiments about the reactions of iodine and iodoacetamide with amino acids and with proteins which do not contain cysteine. The results of the new experiments are given in Table II.

Iodine.—Briefly, iodine can react with cysteine in acid solution and destroy the reducing group. Dilute iodine does not react at pH 3.2 with cystine, tyrosine, or histidine or, so far as is known, with these amino acids

TABLE II

Iodine Consumed by Amino Acids and Cysteine-Free Proteins at 37°C.

Amino acid or protein	Amount iodine added	pH	Time	Iodine consumed
	milliequivalents = *mM* × 2		*min.*	*milliequivalents*
0.001 mM cysteine	0.006	3.2	5	0.006
0.001 mM cysteine	0.010	3.2	30	0.0058
0.001 mM cysteine	0.006	2.2	5	0.006
0.0005 mM cystine	0.0015	3.2	15	0.0
0.001 mM tyrosine	0.008	3.2	15	0.0
0.001 mM histidine	0.008	3.2	5	0.0
0.001 mM tryptophane	0.004	3.2	5	0.004
0.001 mM tryptophane	0.006	3.2	5	0.0041
10 mg. chymotrypsinogen	0.001	3.4	5	0.0
10 mg. chymotrypsinogen	0.002	3.4	5	0.00004
10 mg. gelatin	0.0015	3.2	5	0.00002

in proteins. Iodine does react with free tryptophane in acid solution but does not destroy the reducing group. Furthermore, in the cases studied iodine in acid solution does not react with tryptophane when the tryptophane is part of a native protein.

It has been repeatedly shown that iodine oxidizes cysteine in acid solution and that the SH is oxidized beyond the S–S stage. At pH 3.2 in 0.01 N acetic acid I have found that one molecule of cysteine consumes three molecules of iodine, which corresponds to the formation of cysteic acid, RSO_3H.

Although dilute iodine in acid solution can oxidize cysteine beyond the S–S stage, it does not react with cystine at all in 0.01 N HCl (Dowler, 1928) or, as I have found, in 0.01 N acetic acid. Apparently the first product of the oxidation of cysteine by iodine is not cystine but some

other substance which is oxidized further by iodine more readily than cystine.

The experiments with iodine do not establish the nature of the reactive intermediate product. A likely hypothesis is that RSH is first converted into the free radical, RS (Hellerman, 1937) which can either dimerize to RS–SR or react further with iodine. Schönberg, Rupp, and Gumlich (1933) have given evidence that RS radicals can exist under certain conditions. Another possibility is that RSH is first converted into RSOH (Toennis, 1937).

Theoretically in order to oxidize the free radical RS with the least interference from dimerization to RS–SR one should add an excess of iodine all at once to a dilute solution of RSH. These conditions are not fulfilled in the ordinary iodine titration of cysteine and so less than six equivalents of iodine are used up per molecule of cysteine.

If one molecule of iodine reacts with one molecule of RSH then a free radical is formed not only from the RSH but also from the iodine.

The two step oxidation of RSH to RS–SR and of I^- to I_2 resembles the two step oxidation of dyestuffs discussed by Michaelis and Schubert (1938) in that an apparently trimolecular reaction really consists of successive bimolecular reactions with the intermediate formation of free radicals. In the oxidation of dyestuffs, however, two electrons are removed from a single molecule whereas in the oxidation of RSH and I^- two electrons are removed from two separate particles. Furthermore, the oxidation-reduction systems discussed by Michaelis and Schubert are equilibrium systems and the techniques used to study them are based on this fact. The system formed when iodine is added to cysteine is not an equilibrium system. There are other oxidation-reduction systems formed by the addition of iodine, however, which are equilibrium systems.

On the practical side, it may be possible to use iodine to oxidize the SH of intact protein beyond the S–S stage without any oxidation of the S–S groups present. The S–S groups could then, either before or after hydrolysis, be reduced to SH and estimated without any interference from the original SH groups which have been abolished by the reaction with iodine.

Iodine not only does not react with free cystine in acid solution but does not react with insulin which is rich in cystine.

At pH 3.0 iodine does not react with pure tyrosine or with pepsin which contains tyrosine. In neutral solution iodine converts both pure tyrosine and the tyrosine of pepsin into diiodotyrosine. Diiodotyrosine, like tyrosine itself, reduces the phenol reagent in alkaline solution (Herriott, 1937).

Dilute iodine does not react with histidine in 0.01 N acetic acid.

Iodine reacts with tryptophane in 0.01 N acetic acid. Two molecules of iodine are consumed per molecule of tryptophane which corresponds to the formation of one molecule of diiodotryptophane and two molecules of HI. The tryptophane treated with iodine, like untreated tryptophane, reduces the phenol reagent. At pH 3.4, dilute iodine does not react with native chymotrypsinogen, which seems very rich in tryptophane. Chymotrypsinogen gives 4.3 times as much color as casein with the Bates (1937) modification of the May and Rose tryptophane reaction (Bates, private communication).

In the experiments on the reactions of iodine and amino acids a new colorimetric method for the estimation of iodine is used which can also be applied to the estimation of small amounts of iodide such as are liberated in the reaction between iodoacetamide and SH groups. It has long been known that iodine can be estimated by the blue color it gives with starch provided iodide is added to suppress the dissociation of the starch-iodine complex. I have found that half saturated ammonium sulfate can be substituted for the iodide. When ammonium sulfate is used instead of iodide, iodide can be estimated colorimetrically after it has been oxidized to iodine with iodate. It is, of course, necessary that the iodide solution be free of traces of protein or protein split products which can react with iodine.

Iodoacetamide.—Iodoacetamide reacts with SH groups with the liberation of iodide (Dickens, 1933; Rapkine, 1933).

$$RSH + ICH_2CONH_2 = RSCH_2CONH_2 + HI$$

Iodoacetamide also reacts with amino groups, although more slowly than with SH groups (Michaelis and Schubert, 1934). No other reactions between iodoacetamide and protein groups are known.

DISCUSSION

There are three different theories which are in harmony with the fact that denatured but not native egg albumin gives a nitroprusside test for SH groups.

1. The SH groups of native egg albumin are free and accessible but relatively unreactive.[1] On this basis the SH groups of egg albumin become

[1] By reactivity I mean the observed rate and extent of reaction with a particular reagent under particular conditions. I do not mean the oxidation-reduction potential or the equilibrium with some activated form, neither of which can be calculated from the present experimental data.

It should be emphasized that a given change in the structure of a protein may not

more reactive when the protein is denatured because of changes in the structure of the protein near the SH groups.

Mirsky and Anson (1936 *a*) pointed out that the effect of the denaturation of hemoglobin is simply to extend to the acid side the pH at which the SH groups can be oxidized by ferricyanide.

2. Native egg albumin does not contain any free SH groups. When the protein is denatured SH groups are formed by the breaking of S–S linkages (Burk, 1937) or by the breaking of some as yet unidentified SH linkages (Greenstein, 1938).

3. The SH groups of native egg albumin exist as SH groups but are inaccessible (Mirsky, 1938). When the protein is denatured it is opened up and the SH groups become accessible.

The present experiments on the reaction of iodine and iodoacetamide with native egg albumin and with various amino acids and cysteine-free proteins are most simply explained by the theory that the SH groups of native egg albumin are free and accessible but relatively inactive. The other two theories, which are based on the fact that the SH groups of native egg albumin are not oxidized by ferricyanide, are not definitely disproven. If the other two theories are to explain the new facts about the reactions of native egg albumin with iodine and iodoacetamide, however, they must include additional assumptions for which there is, at present, no independent evidence. The linked SH theory has to assume that iodine and iodoacetamide can react with linked SH groups under the conditions of the egg albumin experiments. The inaccessibility theory has to assume that the interior of the molecule of native egg albumin, although inaccessible to ferricyanide, is accessible to iodoacetamide and iodine. Such a difference is not out of the question since ferricyanide, unlike iodoacetamide and iodine, carries a charge. It should be pointed out, furthermore, that the inaccessibility theory has two advantages. There is independent evidence that the protein molecule opens up on denaturation. And a single explanation is provided for the appearance on denaturation of many different protein groups in more reactive form.

In general, the information at present available does not permit certain conclusions about the exact state of the sulfur groups of native egg albumin. Quite apart from any theory of the state of the sulfur groups of native egg albumin, however, it is now clear at least that, contrary to what was

have the same effect on the ease with which the protein's SH groups are oxidized by ferricyanide as on the ease with which the SH groups are oxidized by some other oxidizing agent or the ease with which the SH groups react with iodoacetamide.

previously supposed, reactions of egg albumin with some SH reagents are possible even when the protein is native. It can also be said that the theory that the SH groups of native egg albumin are free and accessible far from being disproven by the facts, as seemed to be the case, now provides the simplest explanation for the new observations.

Urease and Papain.—Crystalline urease gives a positive nitroprusside test and is inactivated by SH reagents such as copper and mercury salts and iodoacetamide. These results suggest that the activity of urease is dependent on the intactness of SH groups. Despite the facts, however, that urease gives a nitroprusside test and is inactivated by heavy metal salts and iodoacetamide, it is not inactivated by iodoacetate and ferricyanide (Hellerman, 1937).

The apparent contradictions found in the study of urease are now understood. There are SH compounds which react much more readily with iodoacetamide than with iodoacetate (Smythe, 1936). There are SH groups in native egg albumin, as shown in the present experiments, which are not oxidized by ferricyanide and yet are abolished by iodoacetamide. Finally, the activity of urease is not dependent on those relatively reactive SH groups of urease which give the nitroprusside test but on some different relatively unreactive SH groups which do not give the nitroprusside test. If just enough porphyrindin is added to oxidize the SH groups which give the nitroprusside test, the urease is still active and it can still be inactivated by *p*-chlormercuribenzoic acid (Hellerman, 1939).

Balls and Lineweaver (1939*a*) have isolated papain and found that the pure enzyme does not give a nitroprusside test but is inactivated by cystine and iodoacetate. They have further (1939*b*) repeated with papain the kind of experiment which had been done with egg albumin and have found that papain treated with cystine or iodoacetate and then denatured no longer has any SH groups which can be detected with nitroprusside or by iodine titration.

In general, there seem to be in different native proteins and sometimes in a single protein SH groups of different degrees of reactivity. The SH groups of native muscle protein give a nitroprusside test (Arnold, 1911). The SH groups of papain do not give a nitroprusside test but are oxidized by the weak oxidizing agent, cystine. There are SH groups in egg albumin and urease which do not react with nitroprusside or even with ferricyanide but still react with iodoacetamide. Thus for the present one should refer to protein SH groups merely as detectable or not detectable and further specify what reagent is used for the detection and under what conditions it is used.

Application to Viruses.—A few preliminary experiments have been made on the effects of iodine and iodoacetamide on viruses.

5 mg. of tobacco mosaic virus was treated with 1 cc. of 0.0004 N iodine at pH 2.8. The virus after the iodine treatment had roughly its original activity. Further experiments are required to find out just how much the virus was modified by the iodine absorbed, and to find out whether the biological properties of the virus have been changed. In particular it would be interesting to know whether a plant infected by the modified virus produces the original virus.

In previous experiments in which it was shown that the tobacco mosaic virus was modified by chemical means, the virus was inactivated (Stanley, 1938). It has been shown in this laboratory, however, that the enzyme proteins, pepsin (Herriott and Northrop, 1934), carboxypeptidase (Anson, 1937), and chymotrypsin (Kunitz, 1938) can be modified without destruction of the enzymatic activity.

The tobacco mosaic virus and the rabbit papilloma virus are not inactivated by iodoacetamide at pH 8.0. The iodoacetamide was removed by dialysis before the activity tests. It would be desirable to test the effect of iodoacetamide on a virus in more alkaline solution, choosing a virus which is stable in more alkaline solution.

I am indebted to Drs. A. F. Ross and W. M. Stanley for the measurements of tobacco mosaic virus activity and to Dr. R. E. Shope for the measurements of papilloma virus activity.

EXPERIMENTAL

A previous paper (Anson, 1939 *b*) describes the preparation of the solutions of egg albumin, ferricyanide, iodoacetamide, and iodine.

The starch solution is prepared as follows. 2 gm. of Eastman's soluble starch are dissolved in 100 cc. of water. The solution is heated to boiling and then kept boiling 1–5 minutes. This solution is made up anew each day. Various Baker, Merck, and Pfanstiehl starches which were tried either did not give perfectly clear solutions or gave less color with iodine than Eastman's starch. Whether these differences are due to differences in starting material and in manufacturing methods or simply to differences in individual batches I do not know.

Iodine Plus Native Egg Albumin.—Table I shows the amounts of ferrocyanide formed when native egg albumin is treated with iodine under various conditions and then ferricyanide is added in the presence of Duponol PC.

I shall describe first the experiments carried out at pH 6.8. 0.2 cc. of 1 M pH 6.8 sodium phosphate buffer and 1 cc. of iodine solution are added to 1 cc. of 1 per cent dialyzed egg albumin. The solution is allowed to stand for 5 minutes at 37°C. and then there are added 0.3 cc. of 10 per cent Duponol PC and 1 cc. of 0.01 M ferricyanide.

After the solution has stood for 10 minutes at 37°C. the ferrocyanide formed is estimated as already described (Anson, 1939 *b*).

When the experiment is carried out at pH 4.7, 0.2 cc. of 1 M acetate buffer containing equal parts acetic acid and sodium acetate is added instead of the phosphate buffer. Before the addition of Duponol PC and ferricyanide, 3 drops of 0.5 N NaOH and the phosphate buffer are added.

When the experiment is carried out at pH 3.2, 1 cc. of 1 per cent egg albumin is brought to pH 3.2 (as measured by the quinhydrone electrode) with 0.7 cc. of 0.01 N HCl.

In none of the cases can the yellow color of iodine be detected after iodine is added to the native albumin. At pH 3.2 twice as much iodine as the 1 cc. of 0.0015 N iodine used can be added without the appearance of the iodine color. In water solution the color of 1 cc. of 0.0002 N iodine can readily be detected.

Iodoacetamide Plus Native Egg Albumin.—There are added to 4 cc. of 4 per cent dialyzed egg albumin, 2.6 cc. of 0.06 M iodoacetamide, 0.2 cc. of 0.5 N NaOH, 0.7 cc. of water, and 0.5 cc. of a pH 9.0 borate buffer consisting of 8.5 parts 0.4 M sodium borate and 1.5 parts 0.4 N HCl. The solution is allowed to stand 30 minutes at 25°C. To 0.5 cc. of the solution containing 10 mg. of egg albumin there are added 1 drop 0.1 N HCl, 0.2 cc. of 1 M pH 6.3 sodium phosphate buffer, 0.5 cc. of 0.1 M ferricyanide, and 0.6 cc. of 10 per cent Duponol PC. The solution is allowed to stand 10 minutes at 37°C. and the amount of ferrocyanide formed is found to be 0.0006 mM.

If the whole procedure is carried out with the omission of the iodoacetamide 0.001 mM of ferrocyanide is obtained. This shows that the reducing groups are stable at pH 9.0 in the absence of iodoacetamide. The same result is obtained if iodoacetamide is added together with ferricyanide after the neutralization or if iodoacetamide is added to a pH 6.3 Duponol PC solution of denatured egg albumin and ferricyanide is added 1 minute later. This shows that iodoacetamide does not interfere with the ferricyanide reaction in Duponol solution.

If the egg albumin is quarter saturated with ammonium sulfate after it has stood at pH 9.0 only a slight turbidity is obtained, indicating very little denaturation.

If 1 cc. of 0.4 M ferricyanide is added to the 4 cc. of 4 per cent egg albumin instead of the iodoacetamide, about 0.00005 mM of ferrocyanide is formed in 30 minutes at pH 9.0 from each 10 mg. of albumin, again indicating very little denaturation.

Iodine Plus Amino Acids and Cysteine-Free Proteins.—Table II shows how much iodine is consumed when iodine is added under different conditions to various amino acids and to several native proteins which do not contain cysteine. An excess of iodine is added and the amount of iodine left is estimated by the blue color given with starch.

The starch-iodine color reaction is carried out in 10 cc. of solution containing about 1 cc. of 0.001 N I_2, 0.5 cc. of 2 N H_2SO_4, 1 cc. of starch solution, and either 1 cc. of 3.85 M KI (Woodward, 1934) or 5 cc. of saturated ammonium sulfate. KI is used only when the protein is precipitated by ammonium sulfate. The color is read after 5 minutes against an iodine standard or against a blue glass standard which has been calibrated against an iodine standard. When the Klett photoelectric colorimeter is used the blue glass need not match the blue starch-iodine color. When a visual colorimeter and a glass standard are used, it is necessary to insert a color filter such as Corning No. 241 which transmits fairly monochromatic red light.

Reducing Power of Tryptophane Treated with Iodine.—1 cc. of 0.001 M tryptophane (Hoffmann-La Roche) gives the same color with the phenol reagent as the same amount

of tryptophane treated in 0.01 N acetic acid with 1 cc. 0.003 N iodine or 1 cc. 0.007 N iodide. The color reaction is carried out according to the directions of Folin and Ciocalteu (1927). Tryptophane gives the same color as an equivalent amount of tyrosine, which is a check on the purity of the tryptophane used.

SUMMARY

The following experimental results have been obtained.

1. Native egg albumin treated with iodine and then denatured no longer gives a nitroprusside test or reduces dilute ferricyanide in neutral Duponol PC solution.

2. More iodine is needed to abolish the ferricyanide reduction if the reaction between native egg albumin and iodine is carried out at pH 6.8 than if the reaction is carried out at pH 3.2. At pH 6.8 iodine reacts with tyrosine as well as with cysteine.

3. Cysteine and tryptophane are the only amino acids with reducing groups which are known to react with dilute iodine at pH 3.2 The reducing power of cysteine is abolished by the reaction with iodine, whereas the reducing power of tryptophane remains intact. Pepsin and chymotrypsinogen which contain tryptophane but not cysteine, do not react at all with dilute iodine at pH 3.2.

4. Native egg albumin treated with iodoacetamide at pH 9.0 and then denatured by Duponol PC reduces only 60 per cent as much dilute ferricyanide as egg albumin which has not been treated with iodoacetamide.

5. The SH group is the only protein reducing group which is known to react with iodoacetamide.

The simplest explanation of the new observation that the SH groups of egg albumin can be modified by reactions with the native form of the protein is that the native egg albumin has free and accessible but relatively unreactive SH groups which can react with iodine and iodoacetamide despite the fact that they do not react with ferricyanide, porphyrindin, or nitroprusside.

Preliminary experiments suggested by the results with egg albumin indicate that the tobacco mosaic virus is modified by iodine at pH 2.8 without being inactivated and that the tobacco mosaic and rabbit papilloma viruses are not inactivated by iodoacetamide at pH 8.0.

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A DIFFERENTIAL VOLUMETER FOR MICRO-RESPIRATION MEASUREMENTS

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The measurement of volume change, rather than pressure change, has of late come into increasing use in work on cellular respiration. As compared with the more widely used manometric respirometer, the volumetric apparatus, which has no greater complexity, has two advantages. In the first place, volumetric readings can be made at close intervals since there is no need to adjust the vessel each time. This makes the volumeter particularly useful in the study of small or transient changes in the rate of respiration. In the second place, the sensitivity of the volumeter is in practice limited principally by the bore of the capillary, while that of the manometer depends on the size of the experimental vessel, which cannot conveniently be less than at least 5 cc. Thus, of the two types, the volumeter is the more easily adaptable to high sensitivities.

Two types of volumeter have been used in micro-respiration work. The differential volumeter measures the volume change in the experimental vessel as against another vessel which is sealed off from the atmosphere. Thus this type is independent of variations in the barometric pressure. The principles and working of the differential volumeter have been discussed by Fenn (1935). The single volumeter measures the volume change in the experimental vessel as against the barometric pressure, so that a control vessel must be used as a thermo-barometer. The former is the more practical for most purposes.

The first differential volumeter was constructed by Thunberg (1905). Since then several others have been developed, of which that of Fenn (1935) is the most widely used. However, in practice the Fenn volumeter is not much more sensitive than the Warburg manometer, partly because it is difficult to manipulate the index drop unless the capillary is rather coarse, and partly because, since the control vessel is the same size as the experimental vessel, the observed volume change is only half that which could be obtained with an infinitely large control vessel.

Duryee (1936) constructed a differential volumeter which included a mercury piston for controlling the index drop. This apparatus used a very large control flask (300 cc.) but did not provide for the addition of material to the respiring tissue.

Several workers have built volumeters, both single and differential, of high sensitivity, but these were limited to use with special kinds of tissue only. (See the extensive literature cited by Fenn, 1935.) Thus the differential apparatus of Brachet and Shapiro (1937) was designed specially to measure the respiration of small eggs, and the single volumeter of Gerard and Hartline (1934) specially for use with nerves. On the other hand, the so called Cartesian divers developed by Linderström-Lang (1937), though capable of exceedingly delicate respiration measurements, do not allow of the addition of material to the vessel during the experiment.

Principles

It was our purpose to construct a differential volumeter of a robust and practical form, sensitive to very small changes in respiratory rate, and capable of general use with various kinds of tissues and organisms. As an essential feature such an apparatus should permit the addition of material during the course of an experiment. The sensitivity must be as high as possible, but the index drop must be easily manipulated. To make short-time measurements reliable, the evolved CO_2 must be very rapidly absorbed by the alkali.

In the apparatus to be described, the experimental vessel has two chambers, allowing ready addition of material during the run. The capillary is of fine bore, but manipulation of the index drop is made easy by the use of a shunt-tube. The control vessel is as large as is consistent with the attainment of temperature equilibrium, so as not to reduce the sensitivity on that score. The surface of the KOH exposed is large in comparison to the respiring material so that CO_2 absorption is very rapid, and provision is made for shaking. Finally, the movement of the index drop may be easily read with a high degree of accuracy.

Preliminary trials in the construction of an apparatus meeting the above requirements were made by Dr. G. Pincus and Dr. N. Werthessen, to whom we are very much indebted. The apparatus constructed by them has been extensively modified by us, and with the mechanical equipment described below will, it is believed, be found satisfactory for a variety of purposes.

Description of the Respirometer

The apparatus is shown in Fig. 1. It comprises four separate parts which are joined by carefully-ground, close-fitting joints.[1]

The control vessel, A, is an upright bottle with a volume of 40 to 60 cc. At its top is sealed on a capillary (internal diameter about 2 mm.) about 12 cm. long, with a stop-cock just above the top of the bottle. Just above the bottom of the bottle, at right angles to its long axis, the second section is connected by means of a ground glass joint.

The second section, B, consists of a 15 cm. length of fine capillary, selected under the microscope for its small, uniform, circular bore, with a coarser capillary (the shunt-tube)

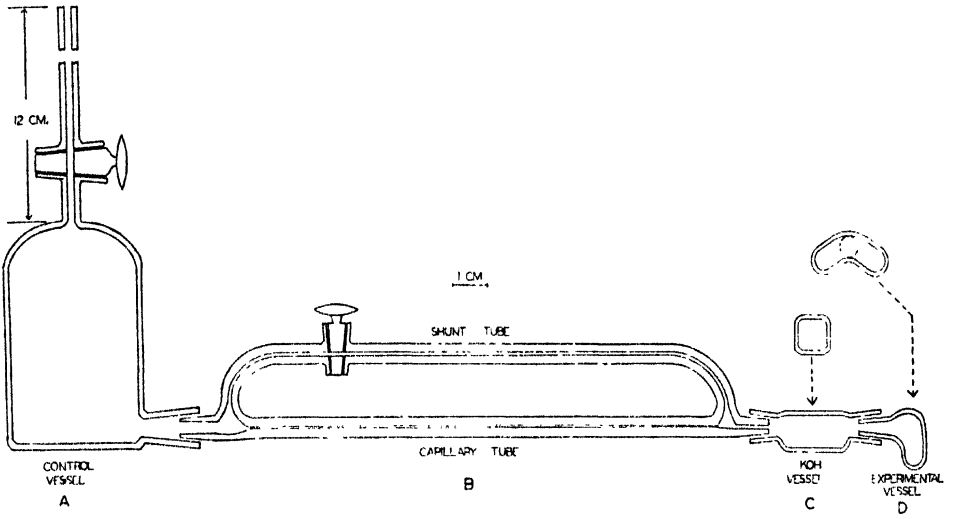


FIG. 1. The differential volumeter. Overall length 29 cm. In assembly, section B is rotated through a small angle so that the shunt-tube does not lie directly above the capillary. The stop-cock in the shunt-tube is also mounted at an angle.

sealed to both ends of it. Each of these junctions continues into the male part of a ground joint, that at the bottle end having a rather wide bore, but that at the opposite end being thickened to an internal diameter not exceeding 1 mm., in order to keep the volume of the experimental vessel at a minimum. Near the bottle end of the shunt-tube is a simple stop-cock, placed at an angle to avoid obstructing the view of the fine capillary from above. The diameter of the fine capillary is 0.2–0.3 mm., while that of the shunt-tube is about 1 mm. A transparent glass scale, accurately ruled in ½ mm. marks, is wired to the outside of the fine capillary.

The third section, C, is a small cylindrical vessel for the KOH. The top is slightly drawn in to reduce volume and the bottom flattened to form an area of about 15 x 20 mm., on which the KOH lies in a thin film.

[1] The vessels may be obtained from Messrs. McAlister and Bicknell, Cambridge, Massachusetts. We are much indebted to Mr. McAlister for his cooperation.

The experimental vessel (section D) consists of two small arms about 12 mm. long and with an internal diameter of about 6 mm. These are united at an angle of about 120°. At the junction they open into a ground tube, perpendicular to the plane of the "V" formed by the arms, which fits into one end of the KOH vessel. To facilitate the introduction of the respiring material, this tube is of the widest possible bore—about 4 mm. The volume of vessel D is about 2 cc. while the volume of the KOH and experimental vessels together is 4 to 5 cc. (In practice the effective volume is reduced by the addition of 0.3 cc. of KOH solution and 0.2 cc. of fluid medium for the tissue.) The experimental vessel may be used in certain positions other than that illustrated in Fig. 1, as will be described below.

Manipulation

The apparatus is easily assembled. Into the capillary tube is inserted, *via* either end, a small drop of distilled kerosene colored with Sudan III. If the readings are to be made in red light (as for work with green plants) the kerosene may be colored blue with Anthraquinone Green G Base (Du Pont Co.). The cock in the shunt-tube being open, the index drop is moved along the length of the capillary several times (by tipping the tube) and finally left in the desired position. The control vessel and the capillary are then joined together. A thin film of vaseline is used in all joints (stop-cock grease is used in the stop-cocks). To prevent slipping, the joints are supplied with hooks, around which rubber bands are fixed.

For measurement of O_2 consumption, a ring of warm paraffin is placed around the inner wall of the KOH vessel just above the flattened bottom. The latter is covered with 0.3 cc. of 10 per cent KOH and the vessel is then joined to the free end of the capillary tube.

The experimental vessel may be used in any one of several positions. Ordinarily, it is held in the position: ⌒. The tissue is placed in one arm and the material to be added during the course of the experiment in the other. When, during the experiment, the addition is desired, the whole apparatus is rotated on its long axis until the material runs into the arm containing the tissue. (The tipping device is described below.) If an exactly quantitative addition is required, the liquid to be added may be placed in a small glass boat which slides from one arm into the other when the vessel is tipped. The experimental vessel may also be held in a semi-inverted position: ⌐. In this case the tissue is placed in the joint of the "V," and the material to be added in the horizontal arm. Other positions may also be used, according to the nature of the tissue and the amount of the material to be added. If desired, the tissue may itself be placed in a glass boat and tipped into a new solution.

When the experimental vessel has been joined to the other parts, the whole apparatus is suspended in a constant temperature water bath to within several centimeters of the top of the control vessel's vertical tube. Both cocks are open when this is done. The apparatus is shaken (for method, see below) and when temperature equilibrium has been reached both cocks are closed. If it is necessary to adjust the position of the index drop, either the control or experimental vessel is slightly warmed (by placing the hand nearby for a moment) until the drop reaches the desired location, and the shunt-tube cock then opened. The index drop will remain motionless until the cock is again closed. This is done when equilibrium has been reestablished, and the readings may then begin. If the drop traverses the entire length of the capillary before the experi-

ment is completed, it may be rapidly returned to the starting position by this same procedure, and the readings recommenced.

Before the apparatus is removed from the water bath, both cocks are again opened. When the respirometer is removed, the KOH and experimental vessels alone need be disconnected and cleaned. The apparatus is then again ready for use. Once clean the capillary need not be recleaned unless the drop shows a tendency to stick.

The capillary is best cleaned with a solution either of trisodium phosphate or of "Calgonite." The grease is first carefully removed with acetone and the hot cleaning solution then sucked through the tube. The tube is finally rinsed by sucking hot distilled water through it, and then carefully dried.

Sensitivity

A respirometer of the dimensions given above is capable of registering extremely small respiratory exchanges. The equation for determining the sensitivity of a differential volumeter (see Fenn, 1935) is:

$$F = b \times \frac{Vc + Ve}{Vc} \times \frac{p - y}{760} \times \frac{273}{273 + t}$$

where Vc is the volume of the control vessel, Ve is the volume of the experimental vessel, p is the barometric pressure, y is the vapor pressure of the solutions in the vessels, t is the temperature in degrees Centigrade, and b is the number of cubic millimeters in 1 cm. of capillary length. F is a factor such that: F times the distance moved by the index drop is equal to the volume change in the experimental vessel.

Substituting the actual values for a respirometer of the above dimensions at a temperature of 22° C. and a barometric pressure of 756 mm.[2] we have:

$$F = b \times \frac{40.36 + 4.43}{40.36} \times \frac{756-17}{760} \times \frac{273}{273 + 22} = b \times 1.073.$$

Hence, for a capillary diameter of 0.26 mm., F = 0.57 c.mm. per cm. of drop motion. Thus, a drop movement of 1 mm., which is easily and accurately discernible on the glass scale, indicates a volume change of but 0.057 c.mm.

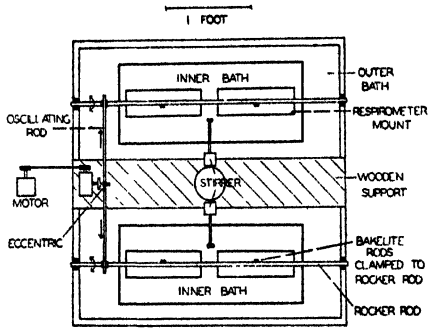
Accessories

Because of its extreme sensitivity, the apparatus must be immersed in a very well controlled constant temperature bath. To accomplish this it is essential to use a double water bath. A bath which will accommodate four respirometers is shown in Fig. 2A.

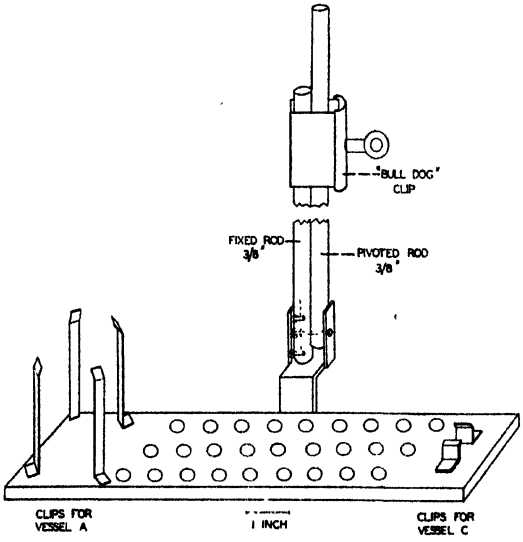
The outer bath is an insulated copper tank 36 × 34 inches, and 24 inches deep. The two inner baths are copper tanks 25 × 10 inches, and 12 inches deep. These are sus-

[2] Normal variations in barometric pressure from day to day make no significant difference in the value of F as long as the ratio of Vc to Ve is large.

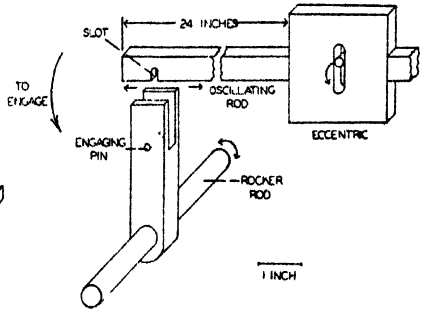
pended in the larger bath by means of bakelite rods. Both inner and outer baths are actively stirred. The propeller shafts leading into the inner baths have bakelite couplings to prevent heat conduction from the stirring motors.



A. Plan of constant temperature tank with inner baths and accessories



B. Brass mount and supports for volumeter



C. Detail of rocking apparatus and release device

FIG. 2

The temperature of the *outer* bath is controlled by means of a sensitive mercury thermo-regulator which regulates (through a relay) a cooling coil and two 100 watt knife heaters. The temperature of the outer bath is thereby maintained with an accuracy of ±0.015°C. If the heating-cooling cycles of the outer bath are regulated to a rather short period (about 10 minutes), the temperature of the *inner* baths will not vary more than ±0.006°C. This variation will have no effect on the movement of the index drop. It is also essential that the stirring of the baths be very rapid since

a small temperature gradient between the two ends of the respirometer will seriously affect the accuracy of the readings.

The respirometer is fixed in a mount made of 1/16 inch brass plate and perforated with numerous holes to permit water circulation (see Fig. 2B). The respirometer is held on the mount by two sets of brass clips together with several rubber bands. The mount is suspended in the inner bath by clamping the (longer) *pivoted* bakelite rod on the ½ inch brass rod which passes over the center of this bath. Ordinary right-angle apparatus clamps will suffice for this purpose. Normally the (shorter) *fixed* bakelite rod is clamped to the pivoted rod by means of a large "Bulldog" paper clip. When the vessel is to be tipped, this clip is removed and the respirometer rotated on its axis by swinging the free end of the fixed rod. When the material has been tipped, the rod is returned to its original position and clamped in place. In this way, tipping is accomplished without any disturbance of the temperature equilibrium and readings may be made immediately.

In order to ensure a rapid gaseous equilibrium within the respirometer it is necessary (in many cases) to shake it. A frequency of about 80 oscillations per minute is adequate. The shaking may be accomplished by producing an alternating rotation in the ½ inch brass rods that are mounted above the centers of the inner baths. The respirometer mounts, which are clamped to these rods, are thereby caused to oscillate in an arc (an amplitude of 2 inches is sufficient) within the bath. The simple apparatus which we have used for producing this type of shaking is illustrated in Fig. 2C. Also figured is a release mechanism for suspending the shaking while readings are being taken.

Since the index scale is ruled in ½ mm. divisions, it is necessary to use a magnifier in taking readings of the drop position. Very accurate readings may be obtained by using a simple projection magnifier. An automobile headlight lamp in a small reflector is suspended over the center of the large tank so that its beam falls on the center of the mounting plate, just below the capillary. At this point is mounted a strip of mirror, 1 × 15 cm., in such a way that the beam of light reflected from it passes through the capillary in the direction of the observer. The transparent glass scale is wired in place above the capillary. Two convex lenses fixed in a water-tight tube can then be arranged to project a magnified image of the capillary and scale on a ground glass screen. In this way, the position of the index drop can be quite easily read to within a tenth of a millimeter.

Typical Results

Fig. 3 illustrates two typical respiration measurements obtained with this apparatus. Curve B shows the change in the rate of oxygen consumption that accompanies the excystment of the protozoan *Colpoda cucullus*. About 5000 freshly encysted organisms were used. The arrow indicates the point at which hay infusion was tipped into the arm containing the cysts. Previous to this point the cysts consumed 0.4 c.mm. of oxygen per hour, and the addition of hay infusion, which causes excystment, resulted in a threefold increase in rate. The curve shows that the apparatus is capable of recording with a high degree of accuracy the changes in the respiratory rate of minute amounts of material. It is also clear that

the possibility of making frequent readings permits of the close following of these changes.

Curve A represents the oxygen consumption of a single section, 3 mm. long, of *Avena* (oat) coleoptile. This is a cylinder with a wall composed of but 6 layers of cells and an outside diameter of about 1.5 mm. The dry weight of such a section is about 0.18 mg. It is evident that the respiration may be easily followed.

More detailed study of these materials will be reported elsewhere. The curves are given here only as examples of the use of the respirometer.

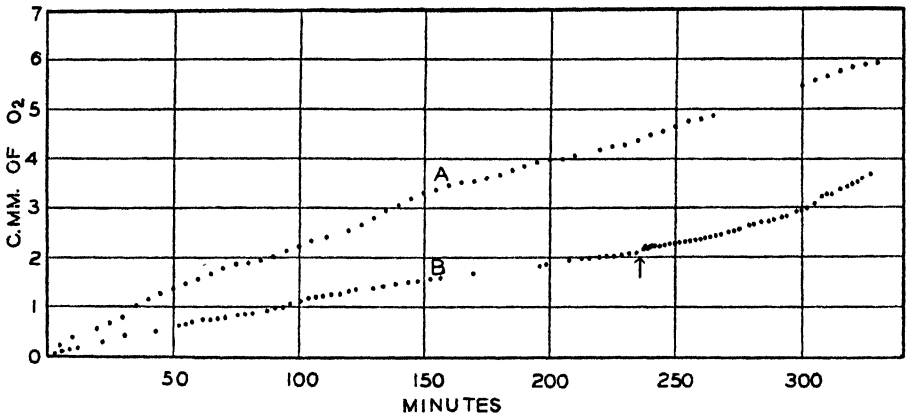


FIG. 3. Sample results obtained showing short interval readings. Curve A, one 3 mm. section of etiolated *Avena* coleoptile in 1 per cent levulose. Curve B, about 5000 cysts of *Colpoda cucullus*. Hay infusion was added at the arrow, excystment beginning some 45–50 minutes after the addition.

SUMMARY

A modified micro-respirometer employing the differential volumeter principle, and mechanical accessories for the same, are described.

The apparatus enables addition of solutions to be made to the biological material during the experiment. It is of a high sensitivity, a drop movement of 1.0 mm. indicating a volume change of about 0.06 c.mm. It may be used with very small amounts of biological material, provides for convenience in manipulation, and allows readings to be made at 1 minute intervals.

Typical results on plant tissue and protozoa are given.

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STABILIZATION OF SPIDER CRAB NERVE MEMBRANES BY ALKALINE EARTHS, AS MANIFESTED IN RESTING POTENTIAL MEASUREMENTS

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INTRODUCTION

If two electrodes are placed upon the uninjured surface of a muscle or nerve and connected by means of a galvanometer, no current will in general flow through the galvanometer. If, however, one of the electrodes is placed upon an uninjured surface and the other upon an injured surface, a current will flow through the galvanometer. This current has been termed the "current of injury" or "demarcation current" and the potential difference between the injured and the uninjured parts, which is associated with the current, has been variously termed, "injury," "demarcation," or "resting" potential.

The current conception of the resting potential is that it is due to a difference of potential between the inside and outside of the fibers which make up the muscle or nerve bundle. The fibers are thought of as being connected in parallel, partly short-circuited by the fluid between them. It is thought that injury permits contact to be made with the inside of the fiber, and the resting potential, which is of the order of tens of millivolts in magnitude, is the resultant of the potentials of the component fibers of the muscle or nerve.

It has been held that the resting potential is due to a difference in concentration of certain ions on opposite sides of a semi-permeable membrane. For an anion impermeable membrane such as is assumed by some for muscle and nerve, the concentration potential may be expressed thus:

$$E = \frac{RT}{F} \ln \frac{C_1}{C_2}$$

where E is the potential in millivolts, R is the gas constant, and C_1 and C_2 are concentrations of the same electrolyte on either side of the membrane.

From the work of Fenn and his coworkers (Hegnauer, Fenn, and Cobb, 1934), it has been calculated (Cowan, 1934) that in frog muscle the concentration of K within the fibers is about eighteen times that in the fluid outside them. Since most of the K within the fibers is believed to be osmotically active, a potential difference of about 73 mv. can be expected from concentration potential formulae. The observed resting potential is considerably lower, about 42 mv. The resting potential observed in crab nerve is also considerably lower than that calculated from concentration potential data (*cf.* Cowan, 1934).

Attempts have been made to lay this discrepancy to (1) short-circuiting by connective tissue and inter-fibrillar fluid, (2) a potential due to asymmetry of the membrane, acting oppositely to the injury potential (Francis, 1937), (3) phase-boundary potentials at the two surfaces of the membrane (Teorell, 1935).

The effect of ions upon the resting potential has long been a subject of interest. In 1905 Höber showed that cations influence the resting potential of frog muscle in the following order of effectiveness: $Li < Na < Mg < Cs < NH_4 < Rb < K$. Anions influence the potential to a much lesser degree, and in the following order of effectiveness: $CNS < NO_3 < I < Br < Cl <$ acetate $< HPO_4 < SO_4$. Netter (1927) found that cations influenced frog nerve resting potentials in the following order: $Ca = Li = Na < Cs < NH_4 < Rb < K$, while anions had no effect.

Höber and Strohe (1929) confirmed the findings of Netter on frog nerve and concluded that the order of effectiveness in general depended upon ionic volume and that deviations from this rule could probably be accounted for by the action of the ions upon the membrane: *e.g.*, changing pore size, charge, etc. They also found that though the alkaline earths had varying influences upon frog nerve resting potentials in themselves, they were able to prevent the depressing action of K, when applied to the nerve simultaneously. More recently Höber and his coworkers have shown that various organic ions and molecules, as well as K, have the power of lowering the resting potential (Wilbrandt, 1937; Höber, Andersch, Höber, and Nebel, 1939).

The experiments here reported were undertaken with a twofold purpose: (1) to investigate the relationship between alkaline earths and K in non-myelinated crab nerve and (2) to determine whether there exists a neutralizing effect of the alkaline earths upon the organic depressants. It will be shown that in crab nerve the alkaline earths have no effect upon the resting potential in themselves but are able to prevent the depressing action not only of K but also of the organic depressants.

Material and Methods

The non-myelinated nerve of the proximal segment of the claw or of the first walking leg of the spider crab, *Libinia canaliculata*, was used throughout for the resting potential

measurements. The nerve was carefully dissected out and permitted to equilibrate in oxygenated sea water for at least a half hour. It was then placed in the nerve chamber and permitted to equilibrate further for about an hour until an approximately steady resting potential value was reached.

The nerve chamber used for the resting potential measurements (Fig. 1) consisted of a box containing paraffin, in which were imbedded at right angles two U shaped glass

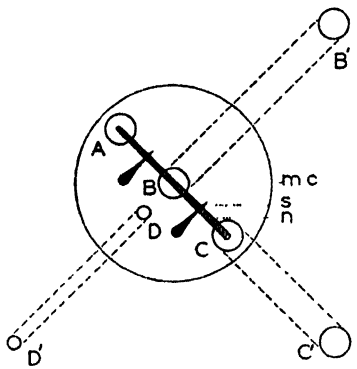

FIG. 1

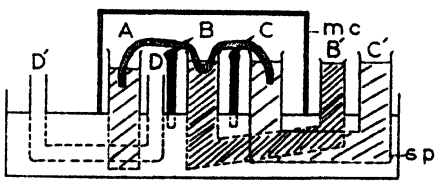

FIG. 2

FIG. 1. Nerve chamber, top view. Dotted line indicates portion of glass tubing below the surface of solid paraffin. A, blind glass tube containing sea water; BB′, glass U tube containing sea water or test solution; CC′, glass U tube containing isotonic KCl; DD′, small glass U tube for air; s, glass support; m.c., moist chamber; n, nerve.

FIG. 2. Nerve chamber, side elevation. s.p., solid paraffin. Other designations as in Fig. 1.

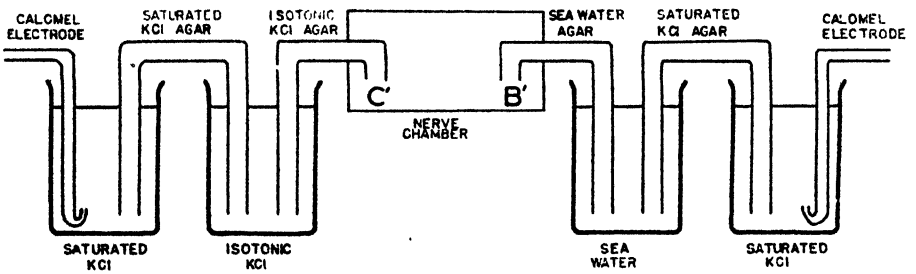

FIG. 3. Electrical set-up for injury potential measurements

tubes, BB′ and CC′ and a blind glass tube, A. The distance (outside measurement) between A and C was about 4 cm.

Tube A was filled with sea water. Tube BB′ was filled either with sea water or some test solution, whose effect upon the nerve was being investigated. Tube CC′ was filled with isotonic KCl.

Two glass supports (s), imbedded in the paraffin, suspended the nerve (n) in such a way that one end dipped into the sea water at A, the middle dipped into the sea water

or test solution at B, and the other end dipped into the isotonic KCl solution at C, which served to injure that end.

Contacts were made at B′ and C′ so that the difference of potential between the middle and injured end of the nerve could be measured. The sea water tube, A, was present only to insure as good a physiological condition as possible in that region, so that no injury effects might pass from that end of the nerve to the middle region of the nerve at B, and to insure that the potential tapped off at B′ and C′ indicated a difference of potential at injured and uninjured portions of the nerve.

Ordinarily, when sea water was in contact with the middle, uninjured portion of the nerve, a difference of potential of about 20 mv. was detected between the middle, uninjured (B) and the injured end portion (C). When the middle portion (B) was killed, the potential difference disappeared.

A moist chamber (m.c.), whose edge was smeared with vaseline, fitted into a circular groove in the paraffin and enclosed the nerve, portions A, B, and C of the tubes, the glass supports (s) and one end, D, of a small U shaped glass tube, DD′, which served to allow air to enter the moist chamber. The glass supports were coated with vaseline and the surface of the paraffin was kept dry to avoid creeping.

Fig. 2 is a side elevation of the nerve chamber.

From the nerve chamber at B′ a sea water-agar bridge led to a beaker of sea water (Fig. 3). A saturated KCl-agar bridge led from the beaker of sea water to a beaker containing saturated KCl, into which dipped the tube of a calomel half cell. From the nerve chamber at C′ an isotonic KCl-agar bridge led to a beaker of isotonic KCl. A saturated KCl-agar bridge, connected the beaker of isotonic KCl with a beaker of saturated KCl, into which the tube of a second calomel half cell dipped. It will thus be seen that the electrical set-up leading off from the middle and injured end of the nerve is symmetrical, and that any potential detected must be due solely to a difference of potential between these two portions of the nerve.

The potential picked off at the calomel half cells was balanced by means of a potentiometer until no deflection was observed in the galvanometer, which was used as a null point instrument. The potential difference could be determined to 0.1 mv. The experimental error was about 0.5 per cent. The curves shown in the various figures represent typical results.

After a few readings had been taken with BB′ filled with sea water, and it had been established that the nerve had reached a fairly steady state, the sea water was removed by means of a pipette inserted at B′, and a test solution, whose influence on the resting potential was about to be investigated was introduced at B′. To prevent contamination the tube BB′ was washed rapidly but repeatedly with the new solution before the next reading was taken.

A number of experiments was also done to determine the effect on excitability of the substances used in studying the resting potential. In these experiments the nerve of the proximal segment of the claw was dissected out but left attached to the more distal portions of the claw. Movement of the claw was used as an index of the excitability of the nerve. The preparation was clamped at the point of articulation of the proximal and next distal segment. The portion of the nerve which had been dissected out was permitted to hang down into a beaker of oxygenated sea water or test solution, as the case might be. At intervals the beaker was removed and the threshold stimulus which elicited movement of the claw was determined by means of an inductorium. Since only gross changes in excitability were being investigated, this method sufficed.

Natural Woods Hole sea water was used throughout. The pH of test solutions used was adjusted with sodium bicarbonate.

RESULTS

The alkaline earths, Ba, Sr, Ca, and Mg, in isotonic solutions of their chlorides (0.29 M), in the great majority of cases have little or no consistent effect upon the resting potential of non-myelinated spider crab nerve (Fig. 4).[1] The resting potential of nerves treated with isotonic solutions of

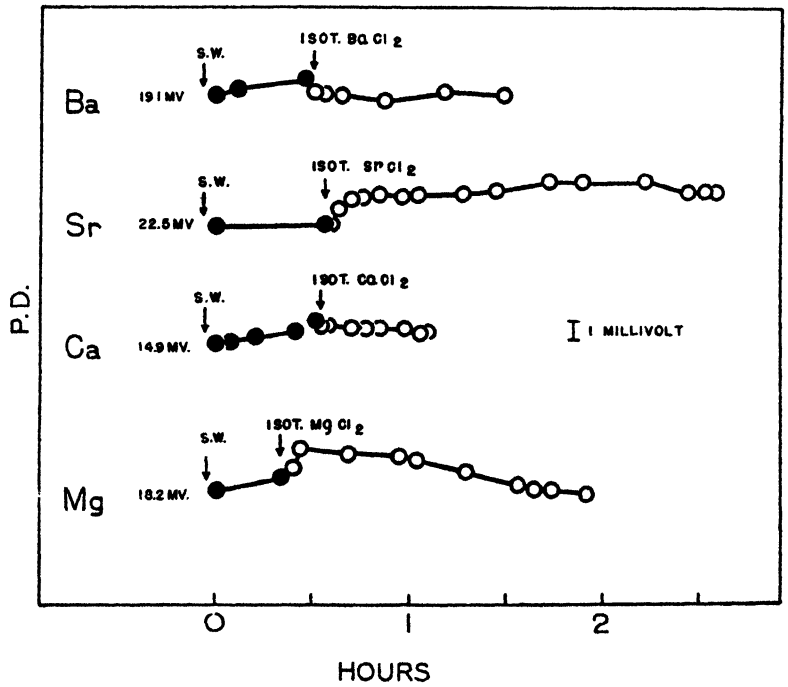

FIG. 4. Absence of any marked effect of alkaline earths upon injury potentials. P.D. in millivolts. The figures to the left of the curves indicate the initial potentials.

these alkaline earths may rise or drop a millivolt or so in an hour or remain constant as compared with the resting potential in sea water. The total change, if any, is not invariably in the same direction, and is probably not significant. If a fall in potential[2] does occur, it is insignificant when com-

[1] In studying the effect of cations upon the electrical impedance of muscle, the author found that while certain ions (Ba, Ca), when applied in isotonic solutions of their chlorides, caused 1000 cycle resistance of muscle to drop, others (Na, K, Mg) had practically no effect upon muscle resistance (Guttman, 1939).

[2] The term "fall in potential" is here used to mean that the P.D. as measured decreases; *i.e.*, approaches zero.

pared in magnitude with the fall in potential of about 1 mv. per minute which follows treatment with isotonic KCl.

The alkaline earths, Ba, Sr, and Ca, have, however, a definite stabilizing influence upon the membrane with the result that K is ineffectual in lowering the resting potential when a solution containing both K and the alkaline earth ion is applied to the nerve. (The term, "stabilization," is here used merely to indicate an alteration of conditions such that substances which were previously strongly depressant no longer have any effect. No particular mechanism is implied.) Ba, Sr, and Ca are not equal in their stabilizing power. A greater proportion of Ca than Sr, and a greater proportion of Sr than Ba is necessary to offset the depressing action of K.

This question was investigated by ascertaining what proportion of isotonic alkaline earth chloride solution had to be added to isotonic KCl in order to bring about neutralization of the K lowering effect. It was found that while a solution containing two parts of isotonic $BaCl_2$ to one part of isotonic KCl resulted in neutralization of the K effect (Fig. 5A), much greater proportions of isotonic $SrCl_2$ and $CaCl_2$ were necessary to effect neutralization. A ratio of five parts of isotonic $SrCl_2$ to one part of isotonic KCl (Fig. 5C), and eleven parts of isotonic $CaCl_2$ to one part of isotonic KCl (Fig. 5E) were necessary before neutralization of the K effect occurred.

That these ratios are threshold ratios for stabilization can be demonstrated, since solutions slightly weaker in alkaline earth content are ineffectual in overcoming the depressing influence of K, *e.g.* while a 5:1 ratio of Sr to K has a neutralizing effect, a 2:1 ratio has not (Fig. 5C); while a 11:1 ratio of Ca to K has a neutralizing effect, a 5:1 ratio has not (Fig. 5E) etc.

That the depressing effect of K would be present in solutions containing such low dilutions of K was shown since the potential dropped when nerves were treated with solutions containing 1/3 isotonic KCl and 2/3 sea water (Fig. 5B); 1/6 isotonic KCl and 5/6 sea water (Fig. 5D), and even 1/12 isotonic KCl and 11/12 sea water (Fig. 5F). (Had the treatment with KCl-sea water solutions been allowed to continue longer, the drop in potential obtained would in every case probably have been even greater, *cf.* Fig. 9.)

It will be noted that the stabilizing power of the alkaline earths: Ba, Sr, and Ca, seems to be related to their place in the atomic table. Whether or not this is of any significance is hard to say. However, from its place in the atomic table, it might be assumed that Mg would have a weaker

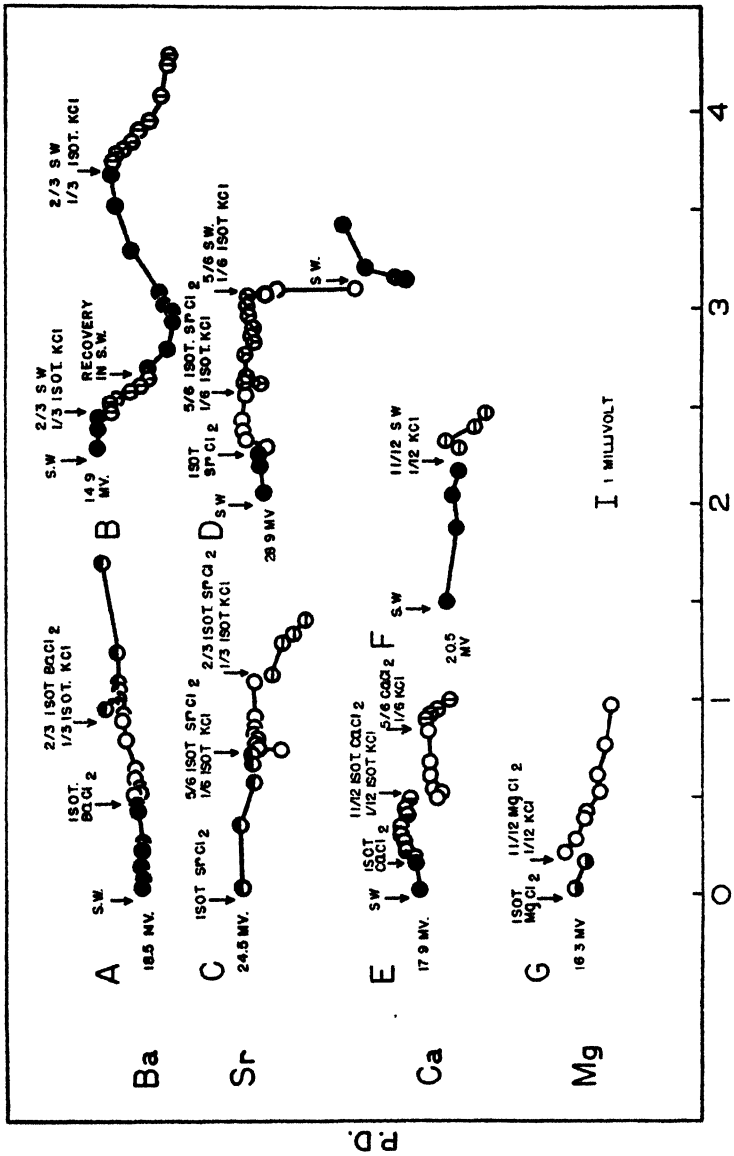

FIG. 5. Alkaline earth neutralization of the effect of K on the injury potential. P.D. in millivolts. Letters A–G indicate different nerves. Figures at left of curves indicate initial potentials.

stabilizing power than even Ca. We can indeed see (Fig. 5G) that a solution containing 11/12 isotonic $MgCl_2$ and 1/12 isotonic KCl is ineffectual in neutralizing the depressing action of K. It might then be suggested that the nerve be treated with a solution higher in Mg content and lower in K content. However, this was not feasible since in solutions weaker in K, *i.e.* containing less than one part of isotonic KCl to eleven parts sea water, the depressing action of the K^+ was not invariably demonstrable.

All conclusions concerning relative neutralizing power of the alkaline earths against the depressing action of K must perforce be somewhat tentative inasmuch as they are based on experiments in which the agents are permitted to act for only short times and the amount of depression of the potential caused by KCl is quite small (Fig. 5). Agents were permitted to act only for relatively short times since it was felt that interpretation would be simpler if irreversible effects were avoided. Later, however, the work was repeated using longer times in order to investigate the duration of the neutralizing effect of the alkaline earths. It was found that the effect may last for many hours. It was also established that the alkaline earths are capable of preventing the action of depressants strong enough to cause, when present alone, a decrease of potential of 50 per cent or more. It will be seen later (in the first experiment presented in Fig. 9) that $BaCl_2$ prevents depression of the potential by KCl for about $2\frac{1}{2}$ hours and that if the same proportion of KCl is mixed with sea water instead of $BaCl_2$ a marked decrease in potential (64 per cent) occurs. The experiments employing longer times lend support to the earlier ones in which only short exposures are involved.

It was shown by Bishop (1932) that veratrine, as well as K, depresses the resting potential of nerve. It was thought that it might be of interest to ascertain whether or not the alkaline earths could neutralize the depressing action of such an organic cation. 5 mg. per cent veratrine sulfate in sea water (0.00004 M) depresses the injury potential of spider crab nerve, yet when this amount is dissolved in isotonic $BaCl_2$, $SrCl_2$, $CaCl_2$ or even $MgCl_2$, the membrane is somehow stabilized (Fig. 6 and *cf.* Fig. 9). The stabilizing power of Mg against veratrine sulfate was of added interest since it had not been possible to demonstrate its stabilizing power against K.

It has been shown by Höber and others (Wilbrandt, 1937; Höber, Andersch, Höber, and Nebel, 1939) that various other organic ions and molecules are able to depress the resting potential of muscle and nerve when applied to the uninjured portion. These include various surface active, lipoid soluble, and cytolytic agents. It was found possible in our

experiments to neutralize the depressing action of some of these agents by means of Ba (Fig. 7).

For example, a 0.01 M chloral hydrate solution in isotonic NaCl (isotonic NaCl, 0.52 M, in itself was found to have little effect upon the resting potential) depressed the potential, but a 0.01 M chloral hydrate solution in isotonic $BaCl_2$ did not (Figs. 7A and 9). (In the first case enough chloral hydrate to make an 0.01 M solution was dissolved in isotonic NaCl, in the

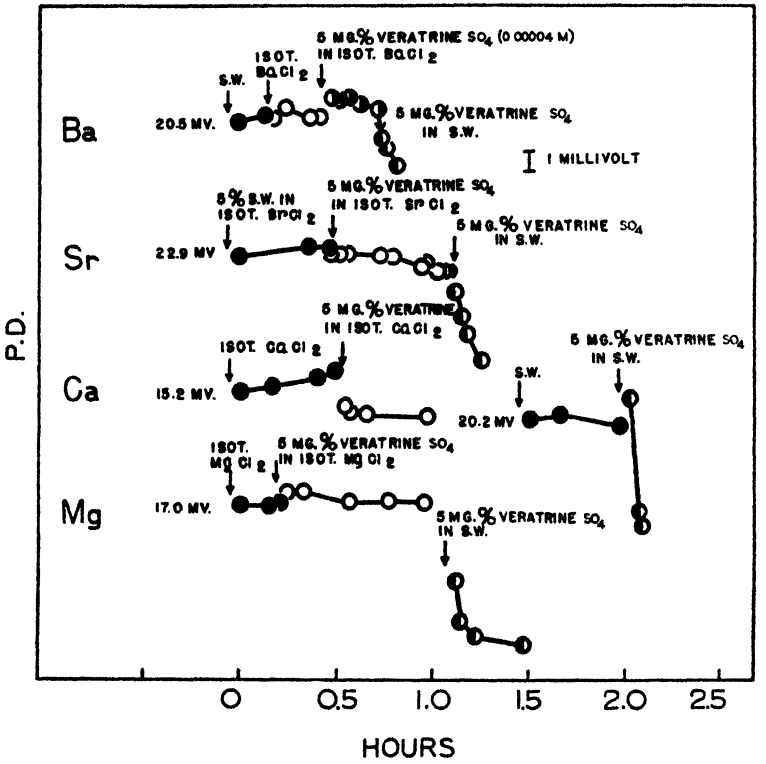

FIG. 6. Alkaline earth neutralization of the depressant effect of veratrine sulfate upon the injury potential. P.D. in millivolts.

second case in isotonic $BaCl_2$.) Likewise a solution of saturated isoamyl urethane in sea water caused the resting potential to fall, while the potential remained at a normal level when the nerve was treated with a solution of saturated isoamyl urethane in isotonic $BaCl_2$ (Fig. 7B and *cf.* Fig. 9). Similarly, 1 per cent saponin in isotonic NaCl (0.014 M) causes a drop in potential, while a 1 per cent saponin in isotonic $BaCl_2$ solution does not (Fig. 7E: see Fig. 9 for 0.1 per cent).

A puzzling situation was at first met with in investigating stabilization of the membrane against the action of Na salicylate. It was found that a solution containing nine parts of 0.5 M Na salicylate to one part of sea

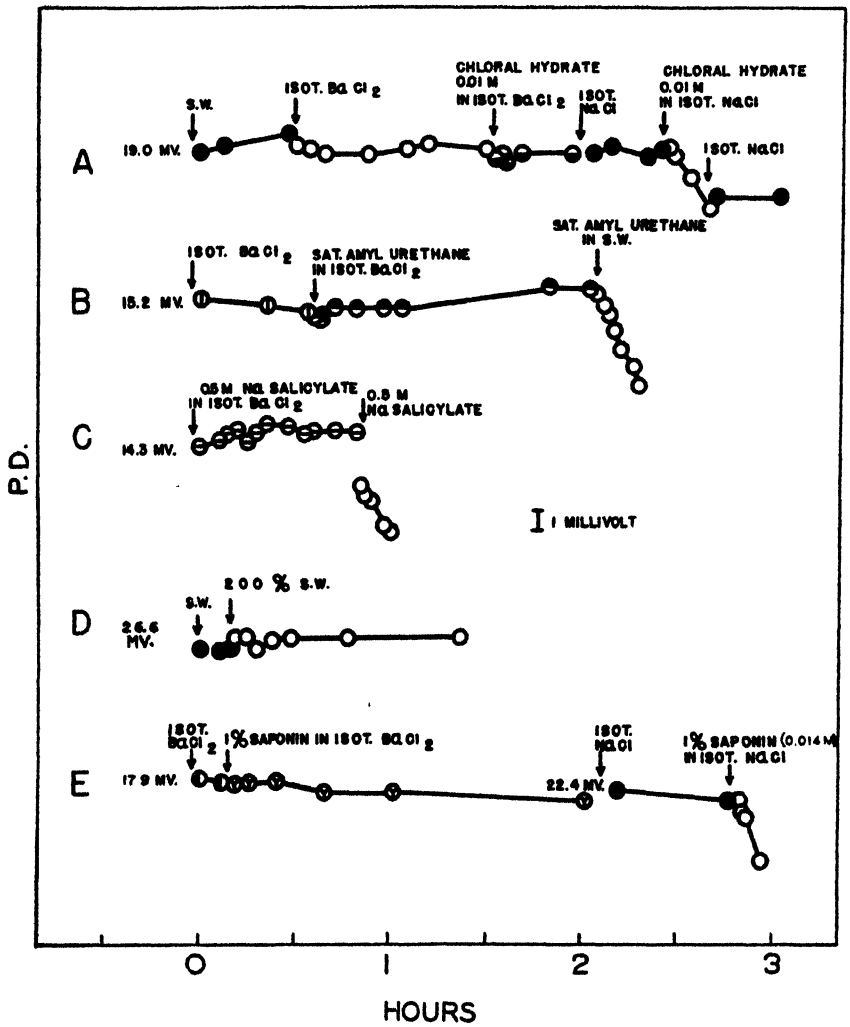

FIG. 7. Barium neutralization of the depressant effect of various organic substances upon the injury potential. P.D. in millivolts.

water was successful in lowering the resting potential, while a solution slightly weaker in salicylate ion, containing eight parts of Na salicylate to two parts of sea water, was not. However, when one part of isotonic

$BaCl_2$ was substituted for the one part of sea water in the former solution (resulting in a solution in which the molar proportions of Na salicylate to $BaCl_2$ are 4.5:0.3), no stabilization took place. A slightly larger proportion of $BaCl_2$ was then employed: 8.5 parts Na salicylate to 1.5 parts isotonic $BaCl_2$ (a solution in which the molar proportions of Na salicylate to $BaCl_2$ are 4.25:0.45) but still no stabilization occurred, presumably due to the relatively insufficient amount of Ba^{++}. However, if the relative amount of $BaCl_2$ was increased slightly at the expense of the Na salicylate, the concentration of Na salicylate became too low to effect a lowering of potential: 8.25 parts of Na salicylate to 1.75 parts sea water caused no depression of the potential. There seemed to be no solution to this problem if one were to confine oneself to the use of isotonic solutions since any isotonic Na salicylate–sea water mixture strong enough in the salicylate ion to depress the potential proved automatically too weak in Ba^{++} for stabilization to take place when isotonic $BaCl_2$ was substituted for the sea water in the mixture.

Finally a strongly hypertonic solution was prepared by dissolving sufficient Na salicylate to make up a 0.5 M solution, not in distilled water but in isotonic $BaCl_2$ (in this solution the molar proportions of Na salicylate to $BaCl_2$ are 5:3). In such a hypertonic solution of Na salicylate in isotonic $BaCl_2$ stabilization was obtained, although in a solution consisting of the same concentration of Na salicylate in distilled water, a sharp drop in potential was observed (Fig. 7C).

The use of a hypertonic solution was justified when it was found that the resting potential was unaffected by sea water as hypertonic as 200 per cent (Fig. 7D). (A 200 per cent sea water solution was made up by dissolving 30 gm. of NaCl in a liter of sea water.)

The lack of effect upon the resting potential of hypertonic sea water was in itself of interest. Wilbrandt (1937) studied the effect of varied osmotic pressure on the potential of frog nerves. He found that if the outer medium were diluted the resting potential rose at first (due presumably to a concentration effect) and then fell. Increasing the concentration of the outer medium, on the other hand, caused the potential to rise. He notes, however, that a similar effect could not be obtained with crab nerves and states that "since we know nothing about the osmotic properties of this nerve it is hard to interpret this negative result."

With the purpose of throwing some light on the osmotic properties of crab nerve, nerves were dissected out and placed in various concentrations of hypo- and hypertonic sea water. At intervals they were removed from the

solutions, blotted rapidly on filter paper, weighed on a torsion balance, and returned to the solution. Errors were largely due to variation in thoroughness of blotting, etc. and were equal roughly to about 10 per cent.

Preliminary experiments indicate that while nerves placed in strongly hypotonic solutions swell appreciably, those placed in solutions as hypertonic as 200 per cent sea water exhibit neither swelling nor shrinking (Fig. 8). The latter observation is of especial interest in view of the fact that no change in resting potential was observed on treatment with such hypertonic sea water solutions.

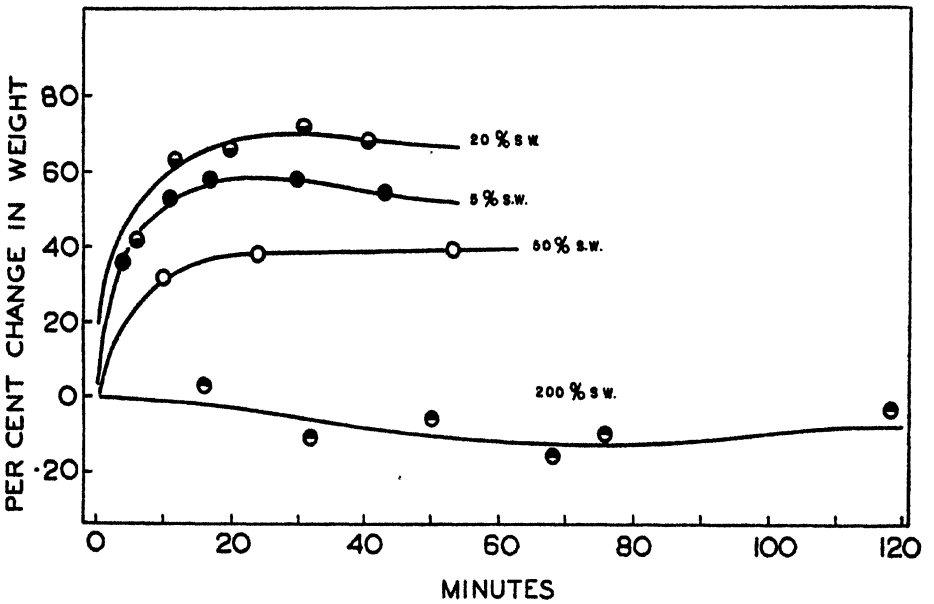

FIG. 8. Osmotic properties of the non-myelinated spider crab nerve. Nerves were immersed in various hypo- and hypertonic sea water solutions, removed, blotted, and weighed on a torsion balance at intervals. Percentage change in weight is plotted against time in minutes.

Experiments involving neutralization of some of the organic depressants by the alkaline earths were recently repeated using longer times of exposure to the agents. The work is summarized in Fig. 9, where it can be seen that the neutralization effect may last for many hours and that the alkaline earths are able to prevent the action of organic depressants strong enough to cause, when present alone, a decrease of potential of 50 per cent or more.

With only one substance, dibutyl amine, was failure encountered in

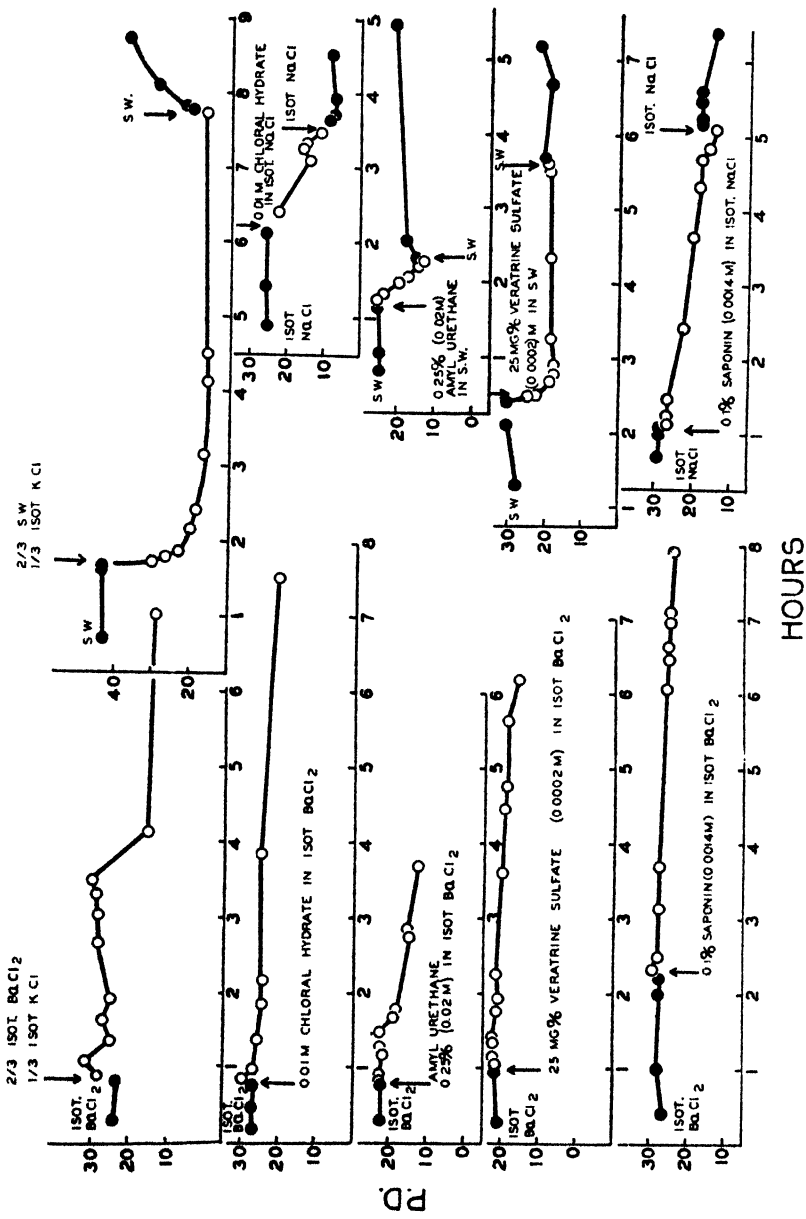

FIG. 9. Barium neutralization of the depressant effect of K and various organic substances upon the injury potential. P.D. in millivolts. Note that the agents were permitted to act for long times.

attempting to obtain stabilization against the lowering effect by means of $BaCl_2$. A solution of 0.2 M dibutyl amine in sea water causes definite lowering of the resting potential (Fig. 10A). There is no indication of neutralization of this effect when a 0.2 M dibutyl amine solution is made up in isotonic $BaCl_2$ instead of sea water (Fig. 10B). Even the gentle falling off of the potential obtained with a 0.15 M dibutyl amine in sea water solution (Fig. 10C), is not completely neutralized when a solution of 0.15 M dibutyl amine

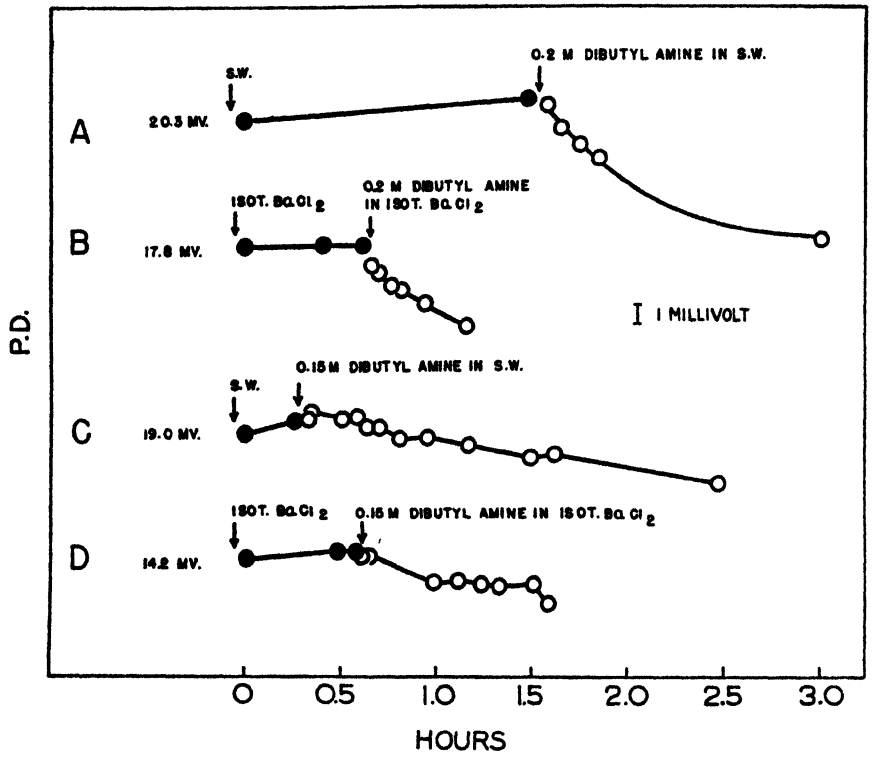

FIG. 10. Effect of dibutyl amine upon the injury potential. P.D. in millivolts

in $BaCl_2$ solution is used (Fig. 10D). As yet no adequate explanation for the lack of stabilization of the membrane by Ba against the depressing effect of the dibutyl amine has been arrived at. In concentrations lower than 0.15 M the lowering effect of the dibutyl amine in sea water upon the resting potential is too poorly marked for investigation at this concentration to be feasible.

Little success was attained in attempting to depress the potential with such substances as solanine in saturated solution; yohimbine, in concentra-

tions as great as 50 mg. per cent in sea water; digitonin, in concentrations as great as 50 mg. per cent in isotonic NaCl; or a 3 per cent cocaine in sea water solution. At times a slight drop in potential could be obtained with these substances; at others, none. It was therefore not feasible to attempt stabilization against the action of these substances.

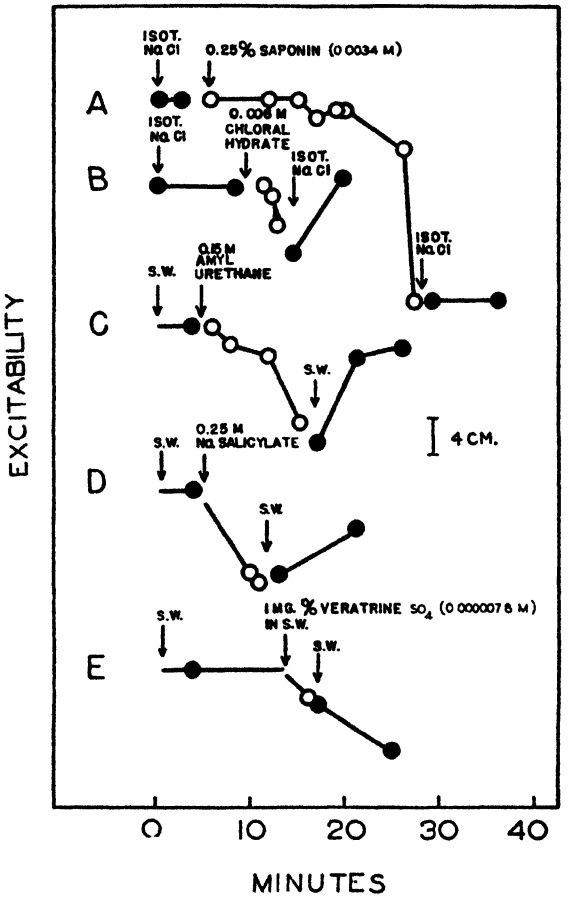

FIG. 11. Depressant effect of certain organic substances upon excitability. ●, nerve in sea water or isotonic NaCl (0.52 M); O, nerve in test solution. Unit equals 4 cm. separation of coils of inductorium. Initial coil separation set for threshold excitation of nerve.

It was felt that it might be of interest in addition to determine the effect of the depressing agents and the neutralizing alkaline earths upon excitability of the spider crab nerve. The results of these investigations are summarized in Figs. 11 and 12.

In confirmation of the work of Tashiro (1917), it was found that chloral hydrate (enough for a 0.006 M solution was dissolved in isotonic NaCl instead of distilled water) reversibly depresses the excitability of the spider crab nerve (Fig. 11B). As he had found with ethyl urethane, it was established that isoamyl urethane (enough for a 0.15 M solution was dissolved in sea water instead of distilled water) is capable of depressing excitability of the nerve reversibly (Fig. 11C). In addition saponin, Na salicylate, and

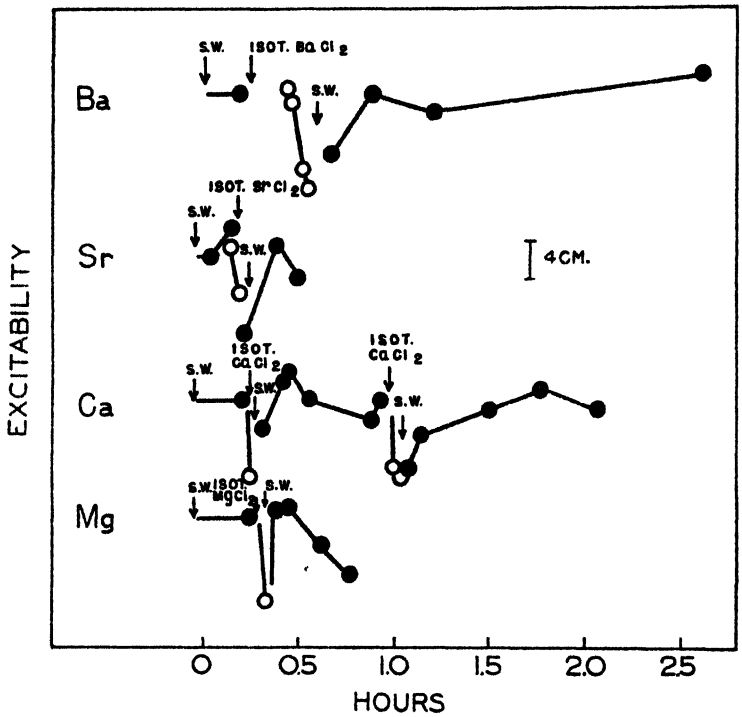

FIG. 12. Depressant effect of alkaline earths upon excitability. Note that the effect is in every case reversible. ●, nerve in sea water; ○, nerve in test solution. Unit equals 4 cm. separation of coils of inductorium. Initial coil separation set for threshold excitation of nerve.

veratrine sulfate were found to depress excitability. In the case of Na salicylate (enough Na salicylate to make a 0.5 M solution was dissolved in distilled water and then one part of this solution was added to one part of sea water resulting in an approximately 0.25 M solution) good recovery was possible if the concentration used was sufficiently low and the time of action sufficiently short (Fig. 11D). In the case of saponin, a strongly cytolytic agent, no recovery was obtained even when a concentration (0.25 per cent dis-

solved in isotonic NaCl or 0.0034 M) so low that its effect was not apparent for several minutes was used (Fig. 11A). No recovery was obtained after use of veratrine sulfate in concentrations as low as 1 mg. per cent dissolved in sea water or 0.0000078 M (Fig. 11E). This lack of reversibility, however, may have been merely a question of not employing the proper concentration over a proper period of time.

The threshold concentration for influencing excitability is much lower than the threshold concentration of the same substance for depressing the resting potential.

In the case of the alkaline earths, Ba, Sr, Ca, and Mg, when used in concentrations as strong as isotonic there was invariably a reversible depression of excitability, provided, of course, that the alkaline earth was not permitted to act upon the nerve for too long a period of time (Fig. 12).

DISCUSSION

It may well be that the measured injury potential is not due to one factor alone, but is the resultant of a number of factors, *e.g.* concentration differences, polarization, and possibly others. It is not at present possible to say whether the agents used in the experiments here reported affect only one of these factors or more than one. However, it might be well to examine some of the theories advanced in explanation of resting potential phenomena.

It has been suggested that the resting potential is largely a concentration potential set up by a difference in K^+ concentration inside the nerve fiber and in its surrounding medium. Cowan (1934) has shown, in support of this theory, that the resting potential may be lowered by increasing the amount of K in a solution in which a crab's nerve is bathed. He argues that increasing the K^+ in the outer medium tends to equalize the amount of K^+ outside and in, and thus the potential difference is lowered. Osterhout was similarly able to depress the resting potential in *Nitella* by increasing the K^+ content of the outer medium (Osterhout, 1931). For a long time it has been felt that normally the rôle of a difference of K^+ concentration is of utmost importance in establishing the resting potential.

It was found by Bishop (1932) for veratrine and by Höber and his coworkers (Wilbrandt, 1937; Höber, Andersch, Höber, and Nebel, 1939) for various other organic ions and molecules that when introduced into the external fluid in which a nerve was bathed, they were efficacious in lowering the potential. What part these or similar substances play normally in nerve physiology, and their possible relation to nerve metabolism are questions of much interest.

There is no reason to assume that because both K and the organic substances depress the resting potential, the mechanism behind their action is identical.

Indeed, the fact that the relative concentration of K necessary to depress the resting potential in crab nerve is of a much greater order of magnitude in general than that of the organic substances suggests the possibility of different mechanisms in the two cases. A 0.04 M KCl solution (one part isotonic KCl to eleven parts sea water) was necessary for lowering the potential perceptibly, while a 0.00004 M veratrine sulfate solution was sufficient for this purpose.

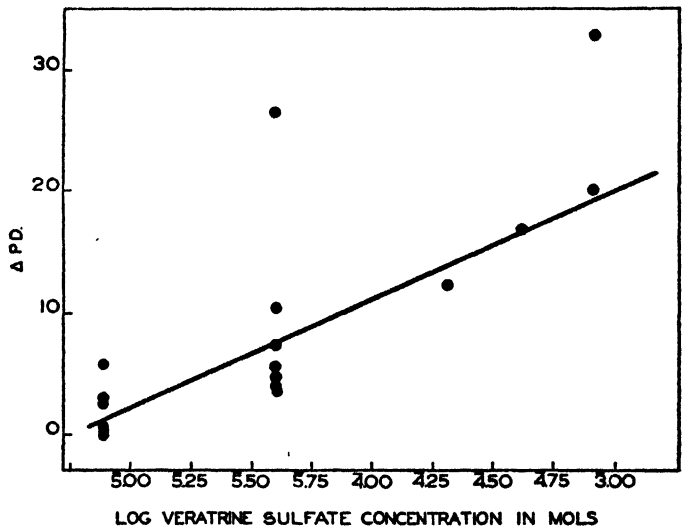

FIG. 13. Effectiveness of various veratrine sulfate concentrations on injury potential. Ordinate indicates drop in potential in millivolts effected by drug after 15 minutes action on nerve.

In an effort to throw some light on the mechanism responsible for the lowering of the resting potential by organic substances, the potential difference drop produced in 15 minutes by veratrine sulfate was plotted against the logarithm of the concentration (in mols) used (Fig. 13). A straight line seems best to express the data, which is of interest since, in general, a concentration effect seems to depend upon the concentration already acting. There is considerable scattering of points but it should be kept in mind that throughout the experiments with almost all agents used, absolute values for the amount of lowering at a single concentration differed widely from nerve to nerve.

The veratrine cation effect resembles an ion effect, such as may be obtained with K. (While there is no direct evidence that the veratrine sulfate effect is an adsorption effect it should be kept in mind that there is often a linear relationship in adsorption phenomena between the amount of adsorption in a given time and the logarithm of the concentration of substance acting.)

Macdonald (1905) altered the concentrations of NaOH, HCl, KCl, and NaCl solutions bathing frog medullated nerve. Over a range of concentrations of 1/8 to 1 molar, he found a nearly linear relation between the resting potential and the logarithm of the K salt concentration in the solution bathing the nerve. More recently Cowan (1934) found that there was an approximately linear relation between the resting potential and the logarithm of the K concentration in a solution bathing crab non-medullated nerve. It is of interest that the linear relation can also be obtained between the resting potential drop and the logarithm of the concentration of veratrine sulfate bathing spider crab nerve in the experiments here reported.

The mechanism behind the depressing action of K and of the organic substances such as veratrine sulfate may or may not be similar. It is striking, however, that it has been found possible, as described above, to annihilate in spider crab nerve the depressing action of both K and these organic substances by adding to the external medium, containing an excess of K or the organic substance, one of the alkaline earths in suitable concentration.

The problem is to account for the stabilizing action of the alkaline earths upon the membrane. At least two types of hypotheses seem to be possible, depending upon one's concept of the nature of the plasma membrane.

Fundamental studies upon the structure of membranes were made by Höber. As early as 1905 he investigated the stabilizing effect of Ba and Sr upon the plasma membrane of frog muscle and described their action as a sort of tanning action, "*Gerbung*," (Höber, 1905). Also Cowan much later (Cowan, 1937) in discussing Solandt's evidence (Solandt, 1936) that Ca increases the speed of accommodation in nerve, points out that this may possibly be explained on the basis of an alteration of the polarizability of the membrane, through an alteration of its permeability to ions of a certain size. He suggests that this might come about by an alteration of the size of pores in a sieve-like membrane.

In opposition to the pore theory of membrane structure, Osterhout has long held that the surface of a cell may be regarded as a non-aqueous phase

immiscible with water, and that potential differences may be considered to arise primarily from diffusion potentials. On this basis he was able to calculate the mobilities of ions in protoplasm and found that guaiacol changes the apparent ionic mobilities of Na and K in various plants (*cf.* Osterhout, 1939).

Osterhout and Hill (1938) also found for *Nitella* that $CaCl_2$ neutralizes the lowering effect of KCl upon the resting potential. They suggest that the Ca effect may be due to a lessening of the partition coefficient of KCl, or to a decreasing of the solubility of an organic substance which sensitizes the cell to the action of KCl.

On the basis of Osterhout's findings it might be suggested that in these experiments on crab nerve the alkaline earths may be decreasing the partition coefficients of KCl and perhaps also of the organic substances, and thus preventing the depressing action of these substances. If it is supposed, on the other hand, that the membrane is a sieve-like structure it might be suggested that the alkaline earths may change the effective pore size and thus neutralize the action of depressants.

An aspect of the results here reported which is of puzzling nature is the fact that relatively large quantities of the alkaline earths are necessary to neutralize the action of the depressing agents. It will be remembered that Jacques Loeb in his classical experiments with *Fundulus* eggs (Loeb, 1906) found that one ion of a bivalent metal was sufficient to render 1,000 molecules of the poisonous salt of a univalent metal harmless. Langmuir and Schaefer (1936) showed that the physical properties of artificial films are profoundly altered by concentrations as low as 10^{-4} M $CaCO_3$ and $BaCO_3$, the rigidity of the film being greatly increased by these agents. A few millimols change in the Ca content of the blood stream is sufficient to upset the delicate ion equilibrium probably present and to bring on parathyroid tetany. It will be readily seen that all these quantities of alkaline earths necessary to offset the activity of other opposing ions or molecules are much smaller in the examples given above than were found necessary to neutralize the depressing action on the resting potential of various agents used in our experiments. This leads us to question whether the same type of "antagonism" can be involved in our experiments as is involved in the other phenomena outlined above.

On the basis of their work with *Nitella*, where Ca neutralized the depressing effect of KCl upon the resting potential, Osterhout and Hill (1938) also stress that the phenomenon is not related to the usual antagonistic effect of Ca upon K inasmuch as the quantities of Ca necessary are too great.

In addition to difference in order of magnitude of quantities involved, there is another significant difference between our findings and the classical antagonism experiments, such as those on *Fundulus* eggs performed by Jacques Loeb. Loeb found that each of the antagonizing ions in itself was poisonous and that together they had no poisonous effect. In the work here reported only one of the agents depresses the potential and when both act together the potential is not depressed. Since the alkaline earths in themselves have no power of lowering the potential, the expression, "antagonism" has been avoided and a more general term; *i.e.*, "neutralization" has been employed.

To summarize, the alkaline earths, which in themselves have no effect upon the injury potential of non-medullated spider crab nerve, are capable of preventing depression of the potential by K and by various organic substances. The phenomenon is not one of antagonism in the sense of Loeb. Two possible explanations for the effect are the following. The alkaline earths may prevent action of the depressants either (1) by altering effective pore size in a sieve-like membrane or (2) altering partition coefficients of the depressants. While the data do no violence to either of two current conceptions of the membrane: ((a) a sieve-like membrane and (b) a water immiscible phase between aqueous solutions), neither do they on the other hand favor either one exclusively.

SUMMARY

1. The alkaline earths, Ba, Sr, Ca, and Mg, in isotonic solutions of their chlorides, have, in general, no effect upon the resting potential of non-medullated spider crab nerve.

2. Ba, Sr, and Ca can, however, prevent the depressing action of K upon the resting potential. The order of effectiveness of these ions in this regard is the following: Ba > Sr > Ca.

3. Ba, Sr, Ca, and Mg oppose the depressing action of veratrine sulfate upon the resting potential. The order of effectiveness is Ba > Sr > Ca > Mg. The relation between drop in potential caused by veratrine sulfate and the logarithm of the veratrine sulfate concentration is a linear one.

4. The action of various other organic ions and molecules which depress the resting potential: saponin, amyl urethane, chloral hydrate, and Na salicylate is neutralized by Ba.

5. Hypertonic sea water solutions do not affect the resting potential. Also, preliminary experiments indicate that the nerves do not shrink in hypertonic solutions although they swell in hypotonic sea water.

6. The alkaline earths depress excitability reversibly. The various organic agents which depress the resting potential also depress excitability, in most cases, reversibly, but the concentrations necessary to depress excitability are much smaller than those necessary to depress the resting potential.

7. The relation of these findings to theories put forward as possible explanations of resting potential phenomena is considered.

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BEHAVIOR OF WATER IN CERTAIN HETEROGENEOUS SYSTEMS*

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In various models, designed to imitate living cells, the behavior of water presents interesting features. If these occur *in vivo*, as appears possible, they may help to explain some of the puzzling aspects of water relations in the living organism.

The living cell is a heterogeneous system since the protoplasm is essentially an aqueous sol or gel with a surface layer which is non-aqueous.[1] In the models the non-aqueous surface layer is represented by guaiacol or by some other liquid immiscible with water.

The experiments fall into 2 groups.

1. Diffusion Experiments.—Solutes diffuse from an aqueous solution *A* through a non-aqueous liquid *B*, into *C* which consists at the start of distilled water. Under certain conditions water moves from *A* to *C*. In some cases the non-aqueous layer, *B*, is stirred mechanically but this does not eliminate diffusion since on each side of each interface there is an unstirred layer through which substances must pass by diffusion.[2] The mechanical stirring of *B* hastens the process by transporting water and solutes rapidly from one of these unstirred layers to the other and presumably affects all the diffusing species alike.

The following types will be described.

Type I: no mechanical stirring (p. 369). In a U-tube (Fig. 1) a non-aqueous liquid, *B*, saturated with water is in contact at one side with an aqueous solution, *A*, and at the other with distilled water, *C*.

Type II (p. 371). As in Type I except that *B* is anhydrous at the start and is stirred mechanically.

* For preliminary accounts see Osterhout, W. J. V., and Murray, J. W., *Science*, 1938, **87**, 430; 1939, **90**, 397; *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 468.

[1] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967; *Tr. Faraday Soc.*, 1937, **33**, 997.

[2] This applies also to stirring by convection. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529. Also Schulman, J. H., and Teorell, T., *Tr. Faraday Soc.*, 1938, **34**, 1337.

Type III (p. 372). As in Type II but *A* and *B* are shaken together and then placed in contact with *C* (distilled water).

2. *Shaking Experiments.*—The movement of water is hastened by shaking together the aqueous and non-aqueous phases until equilibrium is attained. (Diffusion would, of course, accomplish the same thing if sufficient time were allowed.)

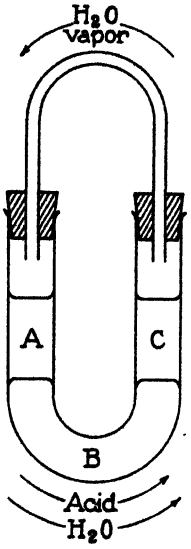

FIG. 1. *A* contains an aqueous solution of trichloroacetic acid. *B* contains guaiacol saturated with water. *C* at the start is water saturated with guaiacol. Acid and water move from *A* through *B* to *C*. In the air above the solutions water vapor moves from *C* to *A*.

The activity of the water was permanently raised by shaking *A* and *B* together. In certain cases where *A* was shaken with *B* and *B* was then shaken with *C*, water passed from *A* to *C* where its activity became higher.

The following types will be described.

Type IV (p. 374). A non-aqueous liquid, *B*, is shaken with an aqueous solution, *A*. After equilibrium is attained the non-aqueous phase is shaken with distilled water, *C*.

Type V (p. 376). A non-aqueous liquid, *B*, is shaken with distilled water, *C*. After equilibrium is attained the non-aqueous liquid is shaken with an aqueous solution.

Methods

Materials.—The materials employed were as follows: Merck's reagent trichloroacetic acid purified by vacuum distillation, Kahlbaum's C.P. guaiacol, Baker's C.P. acetone, Eastman's ethylene chloride, Rossville's absolute ethyl alcohol (gold shield).

Analyses.—In experiments with trichloroacetic acid the total acid was determined by titration with CO_2-free sodium hydroxide, using methyl red as an indicator. When sulfuric acid was present it was determined by the benzidine hydrochloride method of Raschig[3] and the trichloroacetic acid was obtained as the difference. Guaiacol was determined photometrically, using the phenol method of Theis and Benedict[4] with slight modification. The solutions were made up in 10 ml. volumetric flasks and kept at 25° for 30 minutes before measuring. The solutions were measured with the photoelectric densitometer described by Longsworth.[5] A green filter transmitting a narrow band at

[3] Scott, W. W., Standard methods of chemical analysis, New York, D. Van Nostrand Co., 4th edition, 1925, **1**, 507.

[4] Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1924, **61**, 67.

[5] Longsworth, L. G., *J. Bact.*, 1936, **32**, 307.

5600 Å. was used to increase the sensitivity and eliminate errors due to change in the yellow color of the reagent. The filter was made of Schott and Gen filter glasses (2 mm. BG-18, 8 mm. BG-11, and 2 mm. OG-1). A calibration curve was constructed, giving the amount of guaiacol as a function of the optical density using a standard solution containing trichloroacetic acid and guaiacol.

The specific gravity of the solutions was measured with a small picnometer. The water content was calculated by difference.

To obtain the data given in Fig. 7 guaiacol was placed in a weighed and calibrated graduated cylinder and shaken with water. Successive amounts of trichloroacetic acid were added. After each addition, the cylinder was shaken in a thermostat and the two conjugate liquid phases were allowed to separate. The weights and volumes were recorded and measurements were made of the density and concentration of guaiacol and trichloroacetic acid in the aqueous solution. From these data the volume and composition of both phases were computed.

To obtain the data shown in Fig. 4 conjugate solutions were prepared with water, guaiacol, and trichloroacetic acid in equilibrium at 25°C. Portions of about 50 ml. of the guaiacol phase (*B*) were placed in Babcock centrifuge tubes and shaken with about 0.50 ml. of water. The mixtures were brought into equilibrium and allowed to separate at 25°. The volumes were then measured and the concentrations of trichloroacetic acid determined by titration.

Apparatus.—In addition to the U-tube shown in Fig. 1 the following are the types of apparatus employed. All were kept in a water thermostat at 25 ± 0.02°C.

Apparatus No. 2 (Fig. 2) is a U-tube modified to permit greater precision in measuring the volumes of *A* and *C* and to permit the use of larger volumes of *A*. Calibrated tubes (*D*) of 4 mm. bore are attached to the arms and two bulbs of about 10 ml. capacity are inserted in the *A* arm. A capillary stop-cock *E* with a tube *F* for the introduction of mercury is attached to the bottom of the U-tube in order that the aqueous solution may be pushed up into the calibrated tubes *D*.

Apparatus No. 3 (Fig. 3) is essentially a modified U-tube of about 20 mm. bore. To the arms *A* and *C* are attached tubes of about 8 mm. bore containing bulbs of about 12 ml. capacity. Marks *D* are placed on the narrow tubes below the bulbs, and at the 15 and 20 ml. levels above the bulbs are placed marks *E*, measuring from *D*. The bottom part of the U-tube is replaced by a tube of 3 mm. bore in order to reduce the volume of the *B* solution. This tube bears a side arm *F* which is connected to a mercury reservoir *G* permitting the solution in the arms to be pushed up into the narrower graduated tubes for volume measurement. This reservoir is connected to the vacuum line through a 3-way stop-cock to facilitate manipulation. The *B* solution is circulated between the *A* and *C* compartments without breaking the interface by an all glass pump *H*. This pump is actuated by alternate pressure and suction produced by intermittent squeezing of a rubber tube attached to the pump at one end and having the other end plugged. This pump is connected to the lower parts of *A* and *C* through capillary stop-cocks *J* which permit regulation of the rate of circulation and prevent mercury from entering the pump while making volume measurements. The *A* and *C* solutions are stirred by bubbling air through fine capillaries *K*. The air is previously bubbled from a sintered Pyrex disk through a 2 M KCl solution to reduce the volume change from evaporation or condensation. The levels may be regulated by applying back pressure by means of the air trap *L* which may be attached to either arm.

Formation of drops may be seen in B and some of these arrive at the B-C interface: they are not detached from the A - B interface but are formed in the body of B by the same process that causes water to pass from B to C at the interface.

To ascertain whether stirring could detach minute drops of A from the A-B interface and carry them over to C the following test was made. At the start A, B, and C were

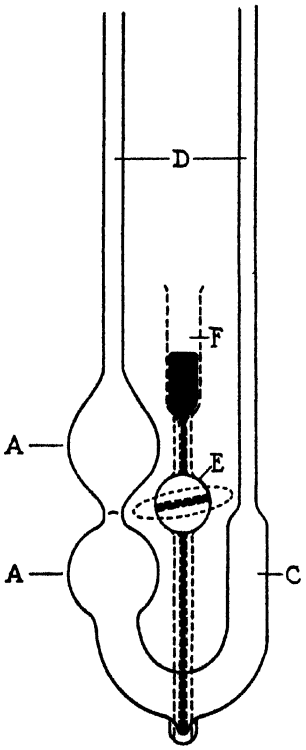

FIG. 2.

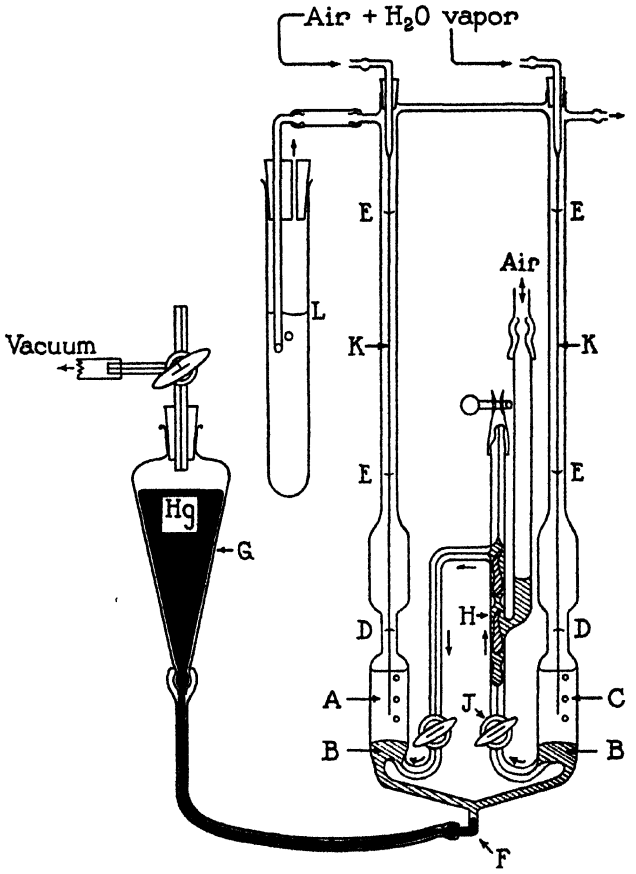

FIG. 3.

FIG. 2. Apparatus No. 2, see p. 367.
FIG. 3. Apparatus No. 3, see p. 367.

brought into equilibrium so that on standing no change occurred in their volumes. Stirring was then started and carried on longer and much more vigorously than in actual experiments. Passage of drops from A to C would be shown by an increase in the volume of C. No such increase was observed although a change of 0.2 per cent could be readily detected.

In some experiments in which A contained sulfuric as well as trichloroacetic acid the sulfuric acid entering C was so much less than the entering water that if the entire movement of sulfuric acid were attributed to the formation of droplets at the A-B interface and their transport in drop form to C we could say that a considerable part of the water entering C did not move in the form of droplets. This is easily shown by calculating the amount of water which would be contained in the supposed droplets. (Regarding the partition coefficient of sulfuric acid see Fig. 5.)

It might be suggested that there is some diffusion in an aqueous film between the guaiacol and the glass. If such a film exists its cross-section must be very small and in consequence diffusion through it must be very inconsiderable.

Losses due to sampling in the shaking experiments are not shown in the tables[6] because the reported figures are adjusted to compensate for them. Thus if one tenth of the solution is taken for analysis the subsequent values for volumes and for millimoles are multiplied by $10 \div 9$.

Unless otherwise stated the experimental errors are approximately as follows. For density 0.2 per cent; for volume 0.2 to 1 per cent; for concentrations: trichloroacetic acid 1 per cent, guaiacol 3 per cent, acetone 1 per cent, water 1 per cent.

In stating the results the figure following the sign $\pm$ refers to this experimental error; it is not the statistical probable error.

In all cases the temperature was 25 ± 0.02°C.

In the following experiments guaiacol was employed as the non-aqueous liquid.

Type I. In a U-tube (Fig. 1) we place guaiacol at B. Resting on the guaiacol at A and C are aqueous solutions which form separate phases in contact with the guaiacol.

The guaiacol is shaken with distilled water until equilibrium is attained and we place in A and C equal amounts[7] of the water which has been shaken with the guaiacol: the system is in equilibrium throughout and no movement of water occurs.

If we now lower the activity of the water in A by adding trichloroacetic acid we might expect water to move from C to A. If this occurs it is presumably only at the start[8] for we find as a rule that after standing for some time water and acid have moved from A to B and from B to C. The higher the concentration of acid in A the greater the movement of water into C.

In order to analyze the solutions we remove all of A and all of C thus

[6] Figures in parentheses in the tables refer to those cases in the non-aqueous phases where the composition was determined by subtracting the amount of each constituent in the corresponding aqueous phase or phases from the total amount of that constituent in the system. Figures in square brackets refer to water which was estimated by subtracting the weights of other constituents from the total weight of the phase.

[7] Unless the volumes are identical the upper surfaces of A and C will not be at the same height and consequently there will be a difference of vapor pressure.

[8] See p. 370.

destroying any gradients which exist in them. We then find that the vapor pressure of water in *C* is higher than in *A*.[9] The gradients in *A* are such that at the upper surface water will have a somewhat lower activity than at the bottom because at the bottom acid is leaving *A*, thereby increasing the activity of the water (see p. 375).[10] The gradients in *C* are such that at the upper surface the activity of the water is greater than at the bottom because acid is entering at the bottom and thereby decreasing the activity of the water (see p. 375). Hence if we compare the upper surfaces of *A* and *C* we see that the difference in the vapor pressure of the water may be higher than the analyses indicate and that in any case the movement of water vapor through the air above the solutions must be from *C* to *A*.

The movement of water in the apparatus can continue for months, ceasing only when *A* and *C* become identical in volume[7] and in composition.

Presumably water moves at first into *A* and then in the reverse direction. Hence the per cent of increase of water in *C* depends on when the experiment is stopped. This may explain the great variation we find in this and other types of experiment and the fact that in some cases there is a loss of water from *C*. (We cannot very well get time curves by taking samples at intervals from *C* since this would stir the contents of *C* and so disarrange the system. We therefore take out all of *C* at the end of each experiment and make analyses.)

To save space we have reported only those experiments which show relatively large gains of water in *C* and it should be understood that in every case a range of smaller values was obtained.

Table I shows the result of an experiment in which *A* consisted of an aqueous solution of trichloroacetic acid, *B* consisted of guaiacol saturated with water, and *C* consisted of water saturated with guaiacol. As *B* and *C* were in equilibrium at the start there was no reason for water to move from *B* to *C* unless forced to do so by the acid diffusing from *A*. This type of experiment will be called Type I *a*.

It is evident that water moved from *A* to *B* and from *B* to *C*: the increase of water in *C* amounted to 44 per cent. Here the volume of *B* was 5 ml. and that of *C* was 1 ml. When the relative volume of *C* was larger the gain

[9] This was tested by means of Barger's method. Barger, G., *Tr. Chem. Soc.*, 1904, **85**, 286. When this shows an increase in the vapor pressure of water in the aqueous phase we conclude that there is an increase in the activity of water in this phase (see also footnote 15, p. 382).

[10] But as the acid leaves the lower surface of *A* the solution in this region becomes lighter and tends to rise.

was less striking. When the volume of *B* was 8 ml. and that of *C* was 20 ml. the highest gain was about 6 per cent.

Type II was set up because it seemed desirable to start with anhydrous guaiacol in *B* so that all the water entering *C* would have to come from *A*

TABLE I[6]

An Experiment (Type I a, Unstirred) Showing Movement of Trichloroacetic Acid and of Water from A through B (Guaiacol) into C (Water)

In 66 hours 23.8 millimoles of water entered *C* and thereby gained in respect to concentration, mole fraction, and activity.[9] The increase of water in *C* was 44 ± 1.1 per cent (Apparatus No. 2).

	A (aqueous)			*B* (non-aqueous)			*C* (aqueous)		
	Start	Finish	Difference	Start	Finish	Difference	Start	Finish	Difference
Volume, *ml.*	10.0	7.9	−2.1	5.0	6.3	+1.3	1.00	1.50	+0.50
Trichloroacetic acid, concn., M	1.64	0.74	−0.90	0.0	1.65	+1.65	0.00	0.42	+0.42
Millimoles	16.4	5.8	−10.6	0.0	10.4	+10.4	0.00	0.63	+0.63
Water, concn., M	47.8	50.4	+2.6	2.9	(11.2)	(+8.3)	54.4	52.1	−2.3
Millimoles	478	398	−80.0	14.4	(70.6)	(+56.2)	54.4	78.2	+23.8
Guaiacol, concn., M.	0.0	0.226	+0.226	8.7	(6.5)	(−2.2)	0.185	0 234	+0.049
Millimoles	0.0	1.79	+1.79	43.3	(41.3)	(−2.0)	0.185	0.351	+0.166

TABLE[6] II

An Experiment (Type II, Stirred) Showing Movement of Trichloroacetic Acid and of Water from A through B (Anhydrous Guaiacol) into C (Water)

In 22.5 hours 116 millimoles of water entered *C* and thereby gained in respect to concentration, mole fraction, and activity.[9] The increase in water in *C* was 10.5 ± 0.6 per cent (Apparatus No. 3).

	A (aqueous)			*B* (non-aqueous)			*C* (aqueous)		
	Start	Finish	Difference	Start	Finish	Difference	Start	Finish	Difference
Volume, *ml.*	20.00	13.35	−6.65	7.0	—	—	20.00	21.06	+4.06
Trichloroacetic acid, concn., M	2.41	0.90	−1.51	0.0	—	—	0.00	0.76	+0.76
Millimoles	48.2	12.0	−36.2	0.0	(17.9)	(+17.9)	0.00	18.3	+18.3
Water, concn., M	[43.6]	[49.8]	[+6.2]	0.0	—	—	55.4	[50.7]	[−4.7]
Millimoles	[871]	[665]	[−206]	0.0	(90.0)	(+90.0)	1107	[1223]	[+116]
Guaiacol, concn., M	0.0	0.20	+0.20	9.1	—	—	0.0	0.19	+0.19
Millimoles	0.0	2.67	+2.67	63.7	(56.4)	(−7.3)	0.0	4.6	+4.6

(*C* consisting of distilled water at the start). When this was tried diffusion was so slow that it became necessary to hasten the process by stirring *B* (see p. 365). Table II shows a result obtained in this way: in 22.5 hours *C* gained 10.5 per cent water, all of which came from *A*.

Type III. In order to minimize changes at the A-B interface, A (trichloroacetic acid in water) is shaken with B (guaiacol) and after equilibrium is attained the non-aqueous phase, now called B', is brought into contact

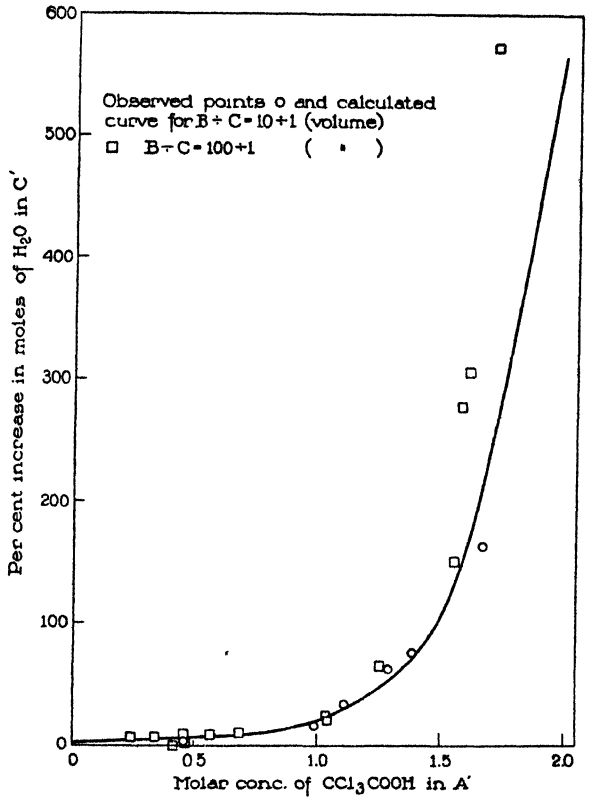

FIG. 4. An aqueous solution of trichloroacetic acid (A) was shaken with anhydrous guaiacol (B) until equilibrium was attained: the resulting aqueous phase is called A' and the guaiacol phase B'. Then 10 parts (by volume) of B' were shaken with 1 part of distilled water (C). Water and acid passed from B' to C and changed C to C'.

The graph showing the percentage increase of moles of water in C', as compared with C, is calculated from Fig. 7. Observed points are shown in circles. The squares show observed points when 100 parts of B' were shaken with 1 part (by volume) of C.

In passing from A to C' the water gained in activity.

with C (distilled water). Water moves into C, producing a large gain. This should be considered in connection with experiments described later (Figs. 4 and 8).

The discussion of these results will be deferred until after the description of the shaking experiments to which we may now turn.

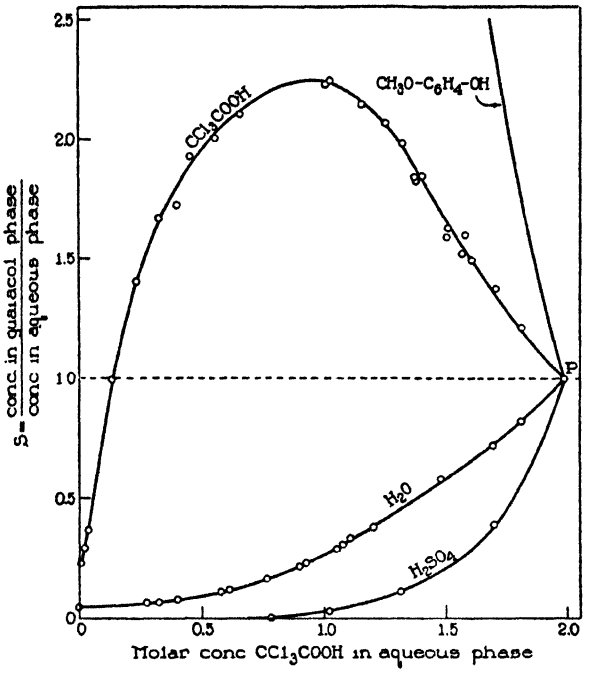

FIG. 5. The partition coefficient, S, equals concentration in the guaiacol phase ÷ concentration in the aqueous phase. As the concentration of trichloroacetic acid in the aqueous phase increases the partition coefficients of water and of sulfuric acid increase up to the plait point[13] (P) where they become unity. The partition coefficient of trichloroacetic acid passes through a maximum and then approaches unity. That of guaiacol decreases to unity at the plait point.

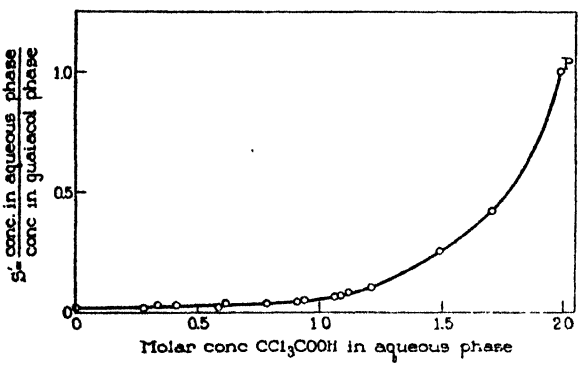

FIG. 6. The partition coefficient S' equals concentration in the aqueous phase ÷ concentration in the guaiacol phase. As the concentration of trichloroacetic acid in the aqueous phase increases the partition coefficient, S', of guaiacol increases up to the plait point[13] (P) where it becomes unity.

Shaking Experiments

These were in all cases carried to equilibrium at 25 ± 0.02°C.

Type IV. We shake A (trichloroacetic acid solution) with B (anhydrous guaiacol) and call the resulting phases A' and B'. After equilibrium is attained we shake B' with C (distilled water) and call the resulting phases B'' (non-aqueous) and C' (aqueous). We then find an increase of water in C' (Fig. 4).

The acid passing from A to B increases the solubility of water in the guaiacol phase (Fig. 5) so that water moves with the acid into B'. When B' is subsequently shaken with distilled water, C, acid passes from B' into C, leaving B' supersaturated with respect to water and the resulting excess of water in B' is taken up by C'. (For the behavior of guaiacol see Fig. 6.)

In such experiments water passes from an aqueous phase (A) where its vapor pressure is relatively low to another aqueous phase (C') where its vapor pressure becomes much higher.[9] This means that the water entering C gains in activity. The activity is regarded as proportional to the vapor pressure at very low concentrations. At higher concentrations we merely assume that as the activity decreases the vapor pressure also decreases without, however, being directly proportional to it.

As the result of the two shakings the original aqueous solutions, A and C, become more nearly alike. There is a net loss of free energy for the system as a whole although there is a gain in free energy on the part of the water which moves from A to C.[11] There is a loss in free energy on the part of the trichloroacetic acid and of the guaiacol.

These statements may be illustrated by Table III. We see that A, consisting of 3.47 M trichloroacetic acid and 37.8 M water, was shaken with anhydrous guaiacol (B) until equilibrium was attained. The resulting aqueous phase, which may be called A', consisted of 1.29 M trichloroacetic acid, 46.5 M water, and 0.44 M guaiacol; the non-aqueous phase, which will be called B', consisted of 2.46 M acid, 19.3 M water, and 3.85 M guaiacol.

Next B' was shaken with distilled water (C) until equilibrium was attained. The

[11] The heat relations are affected by the presence of guaiacol, as is shown by the following experiment.

A solution, A, consisting of 70 ml. of 2.37 M trichloroacetic acid in water was mixed with a stirring rod in a Dewar flask with C, consisting of 16 ml. of distilled water. Both A and C were originally at 25.2°C. On mixing the temperature rose to 25.8°C.

Another portion of A, 70 ml. at 24.9°C., was mixed with B, consisting of 16 ml. anhydrous guaiacol at 25.0°C. On mixing the temperature fell to 23.9°C. The resulting mixture was near the plait point and gave two phases nearly identical in composition. The experiment was repeated with 1 ml. less guaiacol giving a single phase. The fall in temperature was practically the same.

resulting aqueous solution, which will be called C', contained 1.22 M acid, 47.0 M water, and 0.40 M guaiacol. We see that (700 − 432 =) 268 millimoles of water moved from A to C' and in so doing its activity was raised since the vapor pressure of water in C' was higher[9] than in A.

The changes in the activity of the water can be qualitatively described on the basis that increase in concentration, in mole fraction, and in vapor pressure of water in the aqueous solution indicates increase in activity.

On shaking A with B, thereby changing A to A' and B to B', acid passes from A to B and the concentration of water in A rises from 37.8 M to 46.5 M (the mole fraction rises from 0.915 to 0.964). Hence the vapor pressure and activity of the water rise and this applies also to the water which passes into the guaiacol phase, B', since the vapor pressure of the water in B' is equal to that in the aqueous phase, A', in equilibrium with B'.

TABLE[6] III

A (trichloroacetic acid + water) was shaken with B (anhydrous guaiacol) giving A' and B': then B' was shaken with C (water) changing B' to B'' and C to C'. Water moved from A where its vapor pressure and activity were relatively low to C' where they were higher.[9] The gain in moles of water in C' was 62 ± 1.6 per cent (see p. 374).

		A	B	A'	B'	C	B''	C'
	Volume, *ml.*	60.75	31.3	15.1	77.4	7.80	70.1	14.88
	Density, *gm /ml.*	1.248	1.127	1.102	1.226	0.997	1.226	1.097
Trichloroacetic acid	Concn., M	3.47	0.0	1.29	2.46	0.0	2 45	1.22
	Millimoles	211	0.0	19.5	190	0.0	(172)	18 2
Water	Concn., M	37.8	0.0	[46.5]	(19.3)	55.4	(17.7)	[47.0]
	Millimoles	2294	0.0	[702]	(1480)	432	(1240)	[700]
Guaiacol	Concn., M	0.0	9.1	0.44	3.85	0.0	4.08	0.40
	Millimoles	0.0	285	6.7	298	0.0	(286)	5.9

Note: Small discrepancies occur because the values are in some cases obtained by analysis and in others by synthesis or by graphical estimation from the solubility curves.

While the activity of the water rises that of the acid and of the guaiacol falls. As A changes to A' the mole fraction of acid is lowered from 0.086 to 0.027 so that its activity decreases (the vapor pressure of acid in A' and B' must be the same at equilibrium). The activity of the guaiacol is also lowered since its mole fraction in B' (0.15) is much less than in B (1.0).

When B' is shaken with distilled water, C, it might be expected that water would move from C to B': this may happen at the start but as acid moves from B' into C it leaves B' supersaturated with respect to water. The excess water moves from B' into C.

According to Table III the concentration of water becomes higher in C' than in A' (*i.e.* 47.0 M instead of 46.5 M, and the mole fraction becomes 0.966 instead of 0.964). Whether the difference in the concentration of water in A' and C' is exactly 0.5 M is of no importance. It is evident that C' must have a higher percentage of water than A' because shaking B' with water lowers the percentage of acid in B', thereby changing it to B'' which is nearer the base of the triangle on the curve in Fig. 7 than B' is. Hence

B'' is in equilibrium with an aqueous phase C' which has a lower position on the curve than A' and contains more water than A' because it is nearer to the apex of the triangle marked H_2O. This follows from the fact that the tie lines (*e.g.* mn and $m'n'$, Fig. 7) never cross.

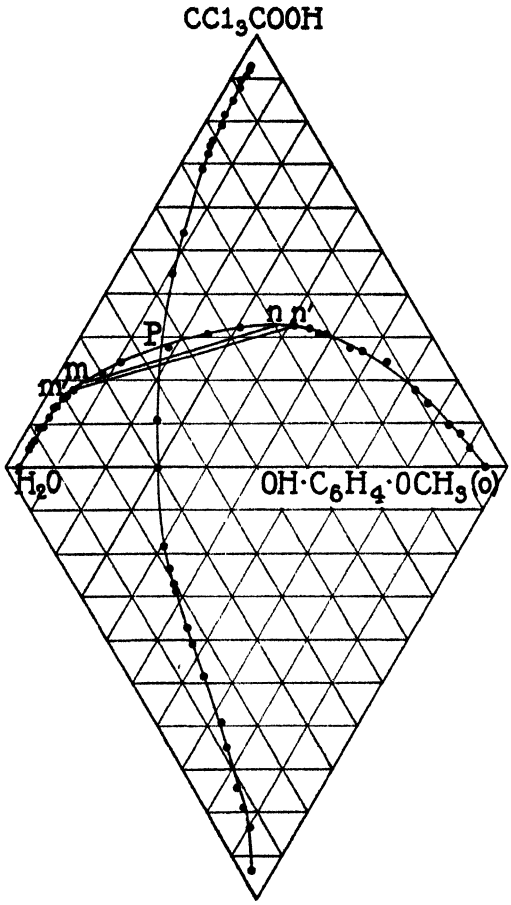

FIG. 7. Mutual solubility of aqueous and guaiacol phases containing trichloroacetic acid. The compositions given in the diagram represent weight percentages. The plait point is at P.[13]

m is an aqueous phase in equilibrium with a guaiacol phase n; m' is an aqueous phase containing more water than m but it is in equilibrium with a guaiacol phase n' containing less water than n and having lower vapor pressure of water. Hence as the mole fraction of water in the guaiacol phase increases the vapor pressure and activity of water in this phase decrease (see p. 381).

Type V. When anhydrous guaiacol (B) is shaken with distilled water (C) and then with trichloroacetic acid solution (A) no water passes from

C to A. Although the guaiacol phase is saturated with water after shaking with C it takes up more water when brought into contact with A because it is altered by the entrance of acid from A.

For example, water and guaiacol are shaken together until equilibrium is attained and the resulting phases are called B_w and C_G (Table IV). The vapor pressure of water, which is the same in both phases, will be called a.

We now lower the vapor pressure of water in C_G from a to a (x), where $x < 1$, by adding trichloroacetic acid until the concentration of acid is 2.04 M and call the resulting solution C_{GA}. We then shake C_{GA} with B_w (guaiacol

TABLE[6] IV

Guaiacol (B) was shaken with water (C) giving the non-aqueous phase B_w and the aqueous phase C_G: To the latter trichloroacetic acid is added, changing it to C_{GA}: this was then shaken with B_w giving B'_w and C'_{GA}. Water passed from C_{GA} to B_w; *i.e.*, from a region where its activity was relatively low to one where its activity was higher.

		B	C	B_w	C_G	C_{GA}	B'_w	C'_{GA}
	Volume, *ml.*	13.7	10.8	14.3	10.3	12.5	18.1	8.8
	Density, *gm./ml.*	1.129	0.997	1.126	0.997	1.158	1.185	1.046
Trichloroacetic acid	Concn., M	0	0	0	0	2.04	1.18	0.58
	Millimoles	0	0	0	0	25.5	21.4	5.10
Water	Concn., M	0	55.4	2.87	54.2	[44.6]	(9.0)	[51.0]
	Millimoles	0	599	41.0	558	[558]	(163)	[449]
Guaiacol	Concn., M	9.10	0	8.65	0.185	0.153	7.19	0.243
	Millimoles	125	0	124	1.91	1.91	130	2.14

Note: Small discrepancies occur because the values are in some cases obtained by analysis and in others by synthesis or by graphical estimation from the solubility curves.

saturated with water) and call the resulting phases B'_w and C'_{GA}: the vapor pressure of water in each at equilibrium may be called y.

The vapor pressure of water in B_w is a, which is greater than the vapor pressure of water in C_{GA} which is a (x). Hence we might expect water to move from B_w to C_{GA}. No doubt this happens at the start but it escapes detection because as acid moves from C_{GA} to B_w it raises the vapor pressure of the water in C_{GA} and lowers it in B_w. When the vapor pressure of the water in C_{GA} becomes higher than in B_w water moves into B_w until at equilibrium the vapor pressure of the water becomes the same in both phases: calling this vapor pressure y we find that the vapor pressure of the water decreases[9,12] in the order $a > y > a$ (x). Hence the net result is a move-

[12] The corresponding concentrations of water in the aqueous phase are 54.2, 51.0, and 44.6 and the mole fractions are 0.997, 0.984, and 0.953.

ment of water from C_{GA} to B_w where the vapor pressure and the activity of the water are higher than in C_{GA}.

Let us now consider the results of other experiments which have yielded the data of Figs. 4–9. The partition coefficient of trichloroacetic acid (Fig. 5) is of interest in that it passes through a maximum. In such ex-

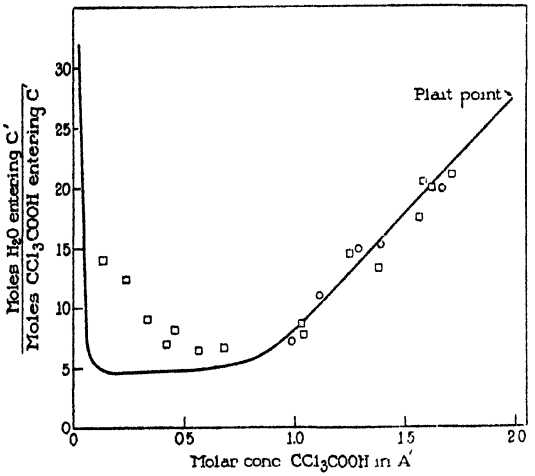

FIG. 8.

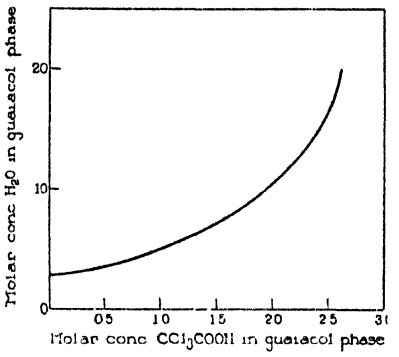

FIG. 9.

FIG. 8. A solution of trichloroacetic acid (A) is shaken with anhydrous guaiacol (B); when equilibrium is attained we call the resulting phases A' and B'. When B' is shaken with distilled water (C), acid, guaiacol, and water pass from B' into C, changing C to C'.

The graph shows calculated increase of the ratio, moles of water entering C' ÷ moles of acid entering C', as the concentration of acid in A' is increased. The graph is calculated from Fig. 7, for the ratio $B' \div C = 10$ and the observed points are shown as circles (○). The observed points for the ratio $B' \div C = 100$ are shown as squares (□). See p. 385. At the left of the minimum there is a difference between observation and calculation, due, in part at least, to the fact that the calculation is made on the basis of $B' \div C = 10$ and the observations are for $B' \div C = 100$.

FIG. 9. The concentration of water in the guaiacol phase increases as that of acid increases (while the concentration of acid in the aqueous phase is rising from zero to 1.3 M). The curve is derived by calculation from Fig. 7 hence no experimental points are shown (but it has been checked by preliminary experiments).

periments the addition of sufficient acid causes the two phases to fuse into one. If this happens to occur at the plait point we have the following situation: the successive additions of acid bring the two phases nearer and nearer together in composition until they become identical and all partition coefficients become unity. The plait point (P) is shown[13] in Fig. 7. When

[13] Regarding the plait point, and the curved vertical line (conjugation curve) which enables us to find any conjugate phase when the phase in equilibrium with it is known,

the plait point is reached at 25°C. *A* contains about 2 M trichloroacetic acid: the weight percentages are, water 55.3 per cent, guaiacol 17.2 per cent, and trichloroacetic acid 27.5 per cent.

It is evident that Fig. 7 enables us to calculate the values in Figs. 4, 5, 6, 8, and 9.

This may be illustrated by an example. In Fig. 7 an aqueous phase *m* is joined by a tie line to a guaiacol phase *n*. The position of *n* in the figure shows that it has the following weight percentages: water 29.5 per cent, guaiacol 37.7 per cent, trichloroacetic acid 32.8 per cent. We now shake 72.4 gm. of *n* (containing 21.4 gm. water) with 6.0 gm. of distilled water thereby increasing the per cent of water in the mixture produced by shaking so that the mixture is nearer to that at the apex of the triangle which is marked H_2O. The mixture is on the tie line connecting *m'* and *n'*, so that on standing the mixture will separate into two phases, *m'* and *n'*. The position of the aqueous phase, *m'*, in the figure shows that it has the following weight percentages: water 76.7 per cent, guaiacol 4.9 per cent, trichloroacetic acid 18.4 per cent. The total weight of *m'* + *n'* is 72.4 + 6.0 = 78.4 gm. and *n'* contains 26.8 per cent water by weight.

We now set up the following equation

$$0.767\,x + 0.268\,(78.4 - x) = 27.4$$

where x = the weight of *m'*, whence x = 12.8 gm. This is the weight of *m'* and since *m'* contains 76.7 per cent by weight of water its water content is 9.83 gm. Since 6.0 gm. of water was used for shaking with *n* it is evident that this 6.0 gm of water has increased to 9.83 gm., an increase of 64 per cent.

Let us now return to the diffusion experiments and consider the effect of substituting various substances.

When we substitute acetic or monochloroacetic acid for trichloroacetic in Types I and II (p. 365) we find little or no increase of water in *C*.

Results like those observed with trichloroacetic acid, but much less striking, were obtained with non-electrolytes; *e.g.*, with acetone. For example, in Type II (p. 365) when *A* at the start contained 20 ml. of 5.8 M acetone, *B* 8 ml. anhydrous guaiacol, and *C* 20 ml. distilled water it was found after 24 hours that the increase in water in *C* was 2.1 ± 0.1 per cent, all of which must have come from *A*. The ratio, moles of water entering *C* ÷ moles of acetone entering *C*, was 0.6.

In Type III (p. 366) larger increases were obtained. For example, after shaking *A* with *B*, *A* contained 20 ml. of 2.18 M acetone + 3.4 M guaiacol, *B* contained 8 ml. guaiacol with 4.18 M acetone and 3.8 M water, *C* contained

see International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 398.

The plait point is the point at which fusion of the two phases occurs without changing the composition of either: at the plait point all partition coefficients become unity. Fusion elsewhere than at the plait point involves changes in composition and the partition coefficients are not unity.

2 ml. distilled water. After 1.4 hours (stirred) the increase in C was 8.0 $\pm$ 0.2 per cent. The ratio, moles of water entering C ÷ moles of acetone entering C, was 1.8.

When water is made to pass from A to C by shaking A with B, thus changing B to B' and B' is then shaken with C (distilled water) increases of water in C may be obtained, amounting to over 400 per cent. Such an experiment showing a gain of 84 per cent of water in C is illustrated in Table V.

The mutual solubilities of water, acetone, and guaiacol are shown in Fig. 10.

TABLE V

A (acetone + water) was shaken with B (anhydrous guaiacol) giving A' and B': B' was then shaken with C (water) changing B' to B'' and C to C'. Water passed from A where the activity of water was relatively low to C' where the activity of water was higher. The gain in moles of water in C' was 84 $\pm$ 1.8 per cent.

		A	B	A'	B'	C	B''	C'
	Volume, *ml.*	34.0	13.8	3.7	43.9	5.96	32.5	17.7
	Density, *gm./ml.*	0.907	1.127	0.969	0.975	0.997	0.985	0.972
Acetone	Concn., M	8.39	0	4.65	6.11	0	6.02	4.51
	Millimoles	285	0	17.2	268	0	196	80
Water	Concn., M	23.2	0	30.3	15.9	55.4	11.6	34.4
	Millimoles	790	0	112	700	330	375	608
Guaiacol	Concn., M	0	9.1	1.09	2.68	0	3.38	0.723
	Millimoles	0	125	4.04	117.8	0	110	12.8

Note: The values for guaiacol in A', B', B'', and C' were obtained by analysis and the corresponding values for water and acetone were then obtained from Fig. 10.

When ethanol was substituted for acetone and ethylene chloride for guaiacol substantial gains in water were found in C in Types II and III (pp. 365–366).

In one case of Type II there were in A 20 ml. of 6.5 M ethanol in water, in B 8 ml. of anhydrous ethylene chloride, and in C 2 ml. of distilled water. After 22.5 hours the increase in water in C was 5.0 $\pm$ 0.1 per cent, all of which came from A. The ratio, moles of water entering C ÷ moles of ethanol entering C, was 0.35.

Similarly in a case of Type III (p. 366) after shaking A and B together A contained 20 ml. 9.1 M ethanol in water, B 8 ml. of 4.3 M ethanol plus 2.5 M water, and C 2 ml. distilled water. After about 4 hours the increase in water in C was 23.5 $\pm$ 0.5 per cent. The ratio, moles of water entering C ÷ moles of ethanol entering C, was 0.51.

It is well known that ethanol increases the solubility of water in ethylene chloride.[14]

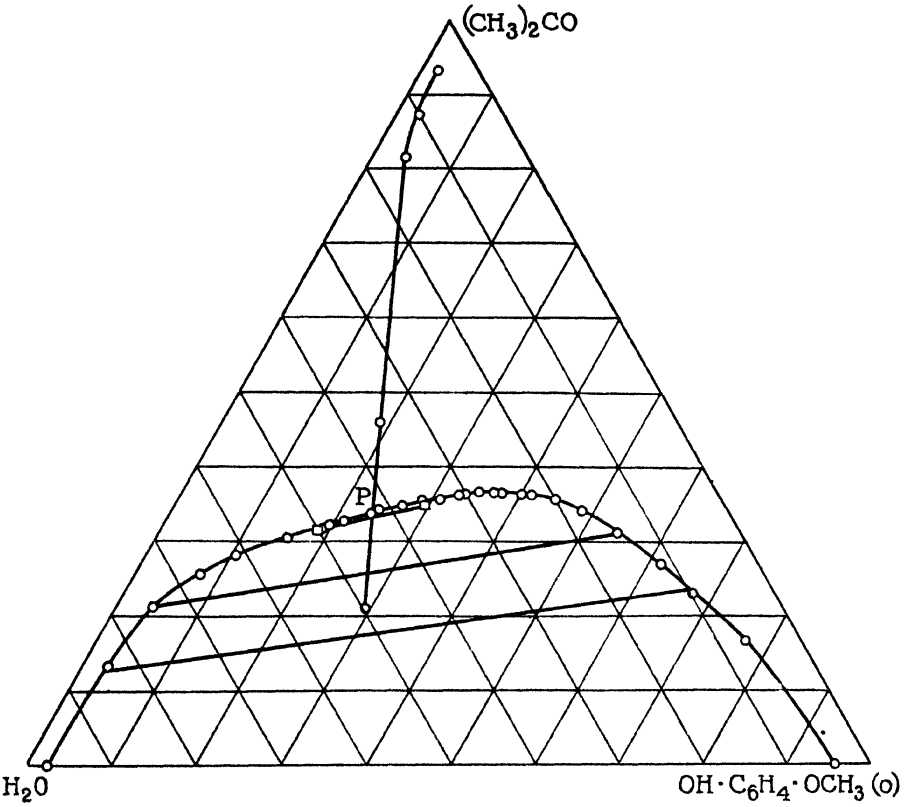

FIG. 10. Shows the mutual solubilities of aqueous and guaiacol phases containing acetone. The compositions given in the diagram represent weight percentages. The plait point is at *P*.[13] Compare with Fig. 7.

DISCUSSION

As the concentration and mole fraction of water increase in the guaiacol phase the vapor pressure, and the activity, of water in the guaiacol phase decrease. This is evident from Table VI and Fig. 11 which show that as the mole fraction of water in the guaiacol phase increases the mole fraction of water in the corresponding aqueous phase decreases. It is also evident from Fig. 7 which shows that as the guaiacol phase takes up more water

[14] *Cf.* International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 411.

and acid and moves along the curve toward the plait point it comes into equilibrium with aqueous phases containing less and less water (*i.e.* further from the H_2O apex of the triangle).

As the concentration and mole fraction of water in the aqueous phase fall off the vapor pressure of water in the aqueous phase falls off.[9] Hence the vapor pressure, and consequently the activity[15] of water in the corresponding non-aqueous phase must also fall off because at equilibrium the vapor pressure of water must be the same in both phases.

A similar result is seen when we substitute acetone for trichloroacetic acid as is evident in comparing Fig. 10 with Fig. 7. The corresponding figure[14] for ethanol, ethylene chloride, and water shows that similar relations obtain here.

TABLE VI

Showing that as trichloroacetic acid is added to the system the mole fraction of water in the guaiacol phase increases and the mole fraction of water in the conjugate aqueous phase decreases. See Fig. 11.

Mole fraction of water	
In aqueous phase	In guaiacol phase
0.996	0.250 (no acid)
0.984	0.518
0.977	0.611
0.966	0.731
0.964	0.754
0.950	0.825
0.939	0.863
0.909	0.909 (plait point)

As the addition of trichloroacetic acid to the water-guaiacol system causes the activity of water in the guaiacol phase to fall off we may say that it falls from a_w to $a_w x$, where $x < 1$. Calling the activity coefficient[16] before the addition of acid f_w we have $f_w = a_w \div c_w$, where c_w is the concentration

[15] At very low concentrations the activity of water in the aqueous phase is approximately proportional to its vapor pressure. At higher concentrations we merely assume that when the vapor pressure falls the activity also falls without being directly proportional.

[16] Before the addition of acid the aqueous phase contains 0.185 M guaiacol and the guaiacol phase contains 2.87 M water. Hence if we adopt the same standard state for both phases the activity coefficient of water in the guaiacol phase is much greater than unity and the same may be said of the activity coefficient of the guaiacol in the aqueous phase.

of water in the guaiacol phase. When enough acid is added to bring the system close to the plait point the concentration of water in the guaiacol phase rises from 2.87 M (the value before acid is added) to 36.5 M, an increase of 12.7 times. Since, as already noted, a_w has fallen to $a_w x$ (where $x < 1$) we now have (calling the present activity of water f'_w) $f_w' = a_w x \div 12.7\, c_w$.

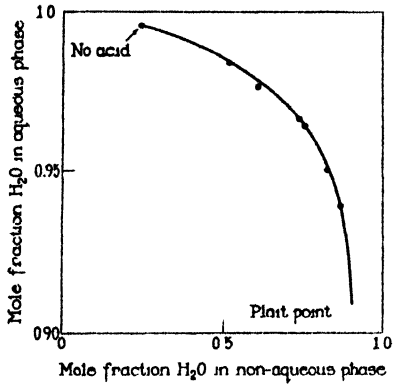

FIG. 11.

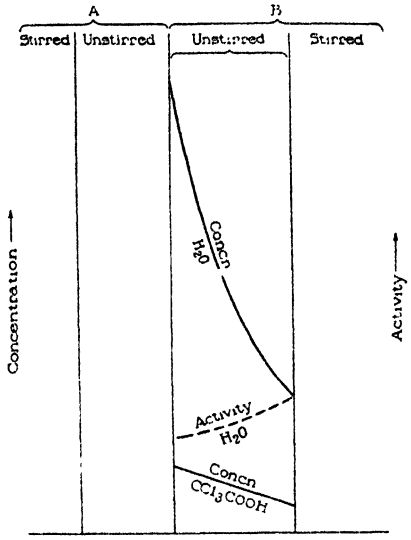

FIG. 12.

FIG. 11. Shows that as trichloroacetic acid is added to the system the mole fraction of water increases in the guaiacol phase and decreases in the aqueous phase in equilibrium with the guaiacol phase. Hence as the mole fraction of water increases in the guaiacol phase the vapor pressure and the activity of water in the guaiacol phase decrease. See Table VI.

FIG. 12. Hypothetical diagram to show molar concentrations of trichloroacetic acid and of water in an unstirred layer of *B*, when a steady state of diffusion of acid has been set up under the conditions of Fig. 9. Concentrations and activities in *B* are plotted as ordinates (which are on a smaller scale for water than for acid). The activity of water in *B* falls off as the concentration of water increases (see p. 375).

This drastic reduction in the activity coefficient of water indicates an attraction[17] of the acid for the water.

We may also infer an attraction[17] of the acid for guaiacol. Fig. 7 shows that as the aqueous phase takes up more guaiacol it comes into equilibrium with guaiacol phases containing less and less guaiacol. Presumably the

[17] To what extent formation of definite compounds occurs must be left open.

activity of guaiacol in the guaiacol phase falls off as its mole fraction falls off, just as happens with water in the aqueous phase. As the activity of guaiacol in the guaiacol phase falls off so does the activity of guaiacol in the conjugate aqueous phase in equilibrium with it. On this basis the activity of guaiacol in the aqueous phase falls off as the concentration and mole fraction of guaiacol increase in this phase. When the concentration has increased from 0.185 M (the value before acid is added) to 1.65 M (the value at the plait point)—an increase of 8.92 times—the value of the activity coefficient of guaiacol in the aqueous phase has decreased to less than $1 \div 8.92$ of what it was before acid was added.

It is the ability to attract both guaiacol and water which enables the acid to bring about the fusion of the two phases when enough acid is added.

Somewhat similar results were obtained when acetone was substituted for trichloroacetic acid (Fig. 10). The corresponding diagram[14] for ethanol, ethylene chloride, and water shows that similar relations obtain here. A number of systems follow this pattern as is evident from the diagrams in the International Critical Tables.[18] Hence we may conclude that a variety of substances[19] act like trichloroacetic acid in attracting water as well as less polar compounds.

The results of the shaking experiments may now be considered in relation to the diffusion experiments.

Let us commence with Type I where a solution of trichloroacetic acid in water, A, is placed in contact with guaiacol saturated with water, B. The shaking experiments show that when these solutions are brought into contact acid passes from A to B (Figs. 4, 7, and 8) and as a result water moves from A to B.

Does this depend on the formation of hydrates? This question arose at the outset but it seemed doubtful that all of the movement of water could be explained in this way. This question was recently taken up by Bent[20] who presented evidence of hydrate formation, but it should be noted that he worked with acetic acid which in our experiments produced little or no movement of water from A to C (see p. 379).

To explain the entrance of water into C as wholly due to definite hydrates

[18] These show that as the non-aqueous phase takes up more water it comes into equilibrium with phases containing less and less water, just as in the guaiacol-water-trichloroacetic acid system. *Cf.* International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 398 *ff*.

[19] *E.g.*, various organic acids, lower alcohols, acetone, phenols, aniline hydrochloride, and pyridine.

[20] Bent, H. E., *Science*, 1938, **88**, 526.

would require the assumption that in the shaking experiments and in the diffusion experiments more than 25 molecules of water are combined in some cases with 1 molecule of acid (Fig. 8).

Since Fig. 8 shows that the ratio, moles of water entering $C \div$ moles of acid entering C, is not constant it would be necessary to assume that the degree of hydration is variable. In that case we might expect the degree of hydration to fall off as the proportion of available water decreased; *i.e.*, as the concentration of acid increased. This is not what occurs as is evident from Fig. 8. Although the curve falls at first it soon passes through a minimum. It then rises which differs from what might be expected if all the water entering C were water of hydration.

The curve, as drawn, was computed from the solubility data given in Fig. 7. At low concentrations it differs from the observed values shown as squares. This is due, in part at least, to the fact that it is made on a different basis. The reason is that more accurate predictions can be made when the volume ratio of $B' \div C$ is taken as 10 but the observation is more accurate when this ratio is taken as 100.

It is evident that at zero concentration of acid no acid can pass into C but some water can do so since the guaiacol is saturated with water and when some of this guaiacol passes into C some water must accompany it: hence the ratio will be infinite. As the concentration of acid increases the curve will pass through a minimum as shown.

The effect of trichloroacetic acid on the activity coefficient of water (p. 382) in the guaiacol phase indicates an attraction between the acid and the water but does not necessarily imply the formation of definite hydrates. A loose association between the water and the acid is all that need be inferred.

Let us now consider the situation at the interface in the diffusion experiments. Although there is always some agitation due to mechanical stirring or to convection there is an unstirred layer on each side of each interface (p. 365). Let us consider such a layer in B adjacent to the A-B interface when a steady state of diffusion of acid has been reached so that the concentration gradient of acid in this layer is linear, as shown in Fig. 12. There will then be a concentration gradient of water if we have the conditions[21] of Fig. 9 for in B there is a definite concentration of water corresponding to each concentration of acid. Although the concentration gradient of acid is due to the flux of acid the concentration gradient of water need not be

[21] At concentrations higher than those shown in Fig. 10 complications ensue as is evident from Fig. 5 which shows that the partition coefficient of trichloroacetic acid passes through a maximum. Above this maximum the addition of trichloroacetic acid to the system causes trichloroacetic acid to move from the guaiacol phase to the aqueous phase.

due to flux of water: there might conceivably be a flux of water in the opposite direction under certain conditions.

If, as already stated (p. 382) the activity of water in *B* falls off as its concentration increases we may represent the activity of water in *B* by the broken line in Fig. 12.

If the conditions of Fig. 9 obtain at the *B-C* interface[22] water may pass under suitable conditions from *B* to *C* if the concentration of acid falls off in this region of *B*, for this region will be left in a supersaturated state with respect to water and since it is at the *B-C* interface some of the excess water may pass into *C*.

Such a falling off in the concentration of acid might occur if a portion of *B* were transported by convection or stirring from a region nearer *A* (a region richer in acid) to the unstirred layer at the *B-C* interface[23] (p. 365). Acid would then diffuse from *B* into *C* and water would follow it if the activity of water in the unstirred layer of *C* were sufficiently lowered by the entrance of acid. The process might resemble that discussed in connection with Type III (p. 372).

The process would be favored by the fact that the acid diffusing into *C* would tend to remain at the bottom of *C* on account of its higher specific gravity. (When *C* is analyzed at the end of the experiment the whole of it is taken so that the concentration of acid at the *B-C* interface is not known.)

The steady state has been discussed because it is useful in clarifying the situation although it does not occur in our experiments. In these the concentration of acid in the unstirred layer of *A* steadily falls (with a consequent rise in the activity of the water). In the unstirred layer of *C* it steadily rises (with a consequent fall in the activity of the water). It might be suggested that this could continue until the activity of water in the latter becomes less than in the former so that water would move from *A* to *C*. In that case analysis might still indicate a lower activity of water in *A* than in *C* since the analysis takes no account of the concentration at the interface because it is made by removing the whole of *A* or of *C* as the case may be. Thus on the basis of the analyses we should say that the water moves from a region of low to one of high activity.

But in order to bring about such a movement of water from *A* to *C* it

[22] It may be questioned whether the concentration of water in *B* will be affected in exactly the same way by acid in flux and by acid at rest. Fig. 9 is based on measurements with acid at rest.

[23] *I.e.* to the surface of the unstirred layer which adjoins the interface.

would seem that the concentration of acid would have to become greater in the unstirred layer of *C* than in that of *A*.

Of course, if hydrates are formed water may enter *C* as water of hydration whenever acid enters *C*.

It is not possible to give a complete picture of the process of diffusion without knowing the concentrations and activities of the diffusing substances in all parts of the system. The difficulties of obtaining this are obvious.

The movement of water in the diffusion experiments recalls the so called "anomalous osmosis" observed with solid membranes. But the latter phenomenon, in the opinion of many investigators,[24] requires the diffusion of electrolytes through pores. In that case the movement of water observed by us would seem to belong to a different category and a different name will therefore be convenient. As the water has the appearance of being carried from a region of lower to one of higher activity by the diffusing substance we suggest the term anaphoresis, from ἀνά (up) and φόρησις (a being borne).

Since in some respects guaiacol acts like certain protoplasmic surfaces[25] it seems possible that similar phenomena may occur in living cells. If so these results have an obvious bearing on the movement of water in the organism and on methods of studying permeability. It becomes necessary to know to what extent a substance entering or leaving the cell carries water with it in the manner here indicated.

In comparing the models with living cells and tissues we should bear in mind that in the organism there is both convection and mechanical agitation (the latter may be due to active or passive motion including protoplasmic streaming).

In some cases the movement of substances in the organism (*e.g.* auxins) appears to present unexpected features. The question may be raised whether these substances are carried along by some other substance as the water in our experiments appears to be carried along by trichloroacetic acid.

Certain puzzling cases of water movement in the organism appear to present some analogy with these phenomena. One of the most striking things about the kidney is the fact that the water moves in a circular path; *i.e.*, most of the water passing from the blood into glomerulus and tubules moves back again into the blood. The analogy with Fig. 1 would be closer if we substituted for the air in the model a membrane more per-

[24] *Cf.* Söllner, K., *Kolloid-Z.*, 1933, **62**, 31.

[25] *Cf.* Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

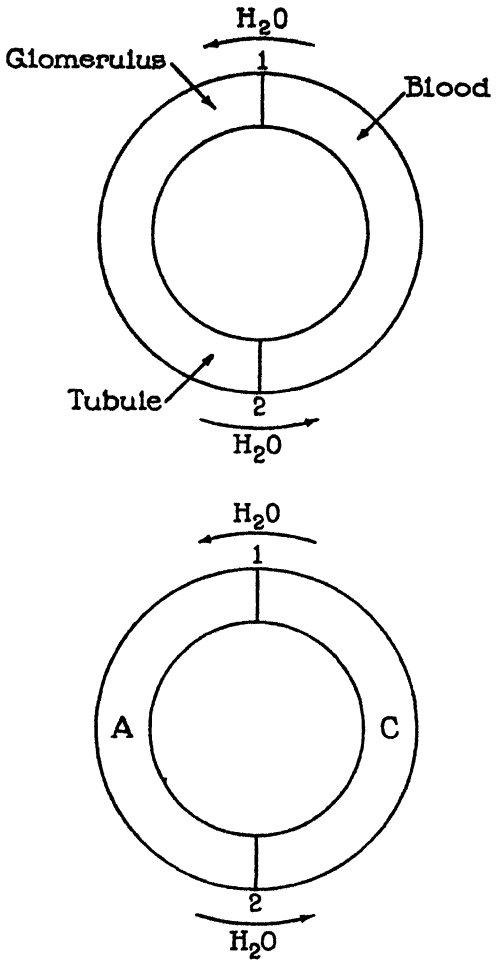

FIG. 13. We assume that the model shown in Fig. 1 is modified by substituting for the air above the solutions a membrane more permeable to liquid water than to acid and to guaiacol: this membrane is called 1. The guaiacol is called membrane 2. The water would then take a circular path, passing through both membranes and if the trichloroacetic acid in *A* were renewed from time to time this would go on indefinitely.

In the kidney the water also takes a circular path. Corresponding to membrane 1 in the kidney is the wall or "membrane" between the blood and the glomerulus; corresponding to membrane 2 is the wall or "membrane" between the tubules and the blood.

meable to liquid water than to acid and to guaiacol. Calling this membrane 1 and the guaiacol membrane 2 we have the situation shown in Fig. 13. Corresponding to membrane 1 in the kidney is the wall or "membrane"

between the blood on one side and glomerulus and tubule on the other: corresponding to 2 is that between the tubule and the blood. In both cases the water pursues a circular path, passing first through one membrane and then through the other.

There are, of course, great differences between the kidney and the model, *e.g.* the kidney does work and requires energy and the passage of water from the blood to the glomerulus is believed to depend largely on hydrostatic pressure. But the kidney resembles the model in that the circular path of the water is a striking feature.

SUMMARY

In various models designed to imitate living cells the surface of the protoplasm is represented by guaiacol which acts in some respects like certain protoplasmic surfaces. The behavior of water in these models presents interesting features and if these occur *in vivo*, as appears possible, they may help to explain some of the puzzling aspects of water relations in the living organism.

When sufficient trichloroacetic acid is added to a two-phase system of water and guaiacol the two phases fuse into one. The effect of the acid is due to its attraction for water and for guaiacol. This is shown by the following facts.

During the addition of the acid the mole fraction of water in the guaiacol phase increases but the activity of water in the guaiacol phase falls off. The activity coefficient of water may fall to less than one twelfth the value it had before acid was added.

The behavior of guaiacol presents a similar picture. During the addition of acid the mole fraction of guaiacol in the aqueous phase increases but the activity of the guaiacol in the aqueous phase presumably decreases. Its activity coefficient calculated on this basis may fall to about one ninth of the value it had before the acid was added.

Somewhat similar results are obtained when acetone is substituted for trichloroacetic acid or when ethanol is substituted for trichloroacetic acid and ethylene chloride for guaiacol.

As trichloroacetic acid increases the mutual solubility of guaiacol and water we find that guaiacol saturated with water and having a high vapor pressure of water can take up water from an aqueous solution of trichloroacetic acid with a low vapor pressure of water: acid passes from the aqueous to the guaiacol phase, thus raising the vapor pressure of water in the aqueous phase and lowering it in the guaiacol phase.

Diffusion experiments present some interesting features. When an aque-

ous solution, A, of trichloroacetic acid is separated by a layer of guaiacol, B, from distilled water, C, under certain conditions water moves from A to C. This depends on the fact that acid moves in the same direction and appears to carry water with it. Similar but less striking results were obtained with acetone diffusing through guaiacol and with ethanol diffusing through ethylene chloride.

These phenomena differ from "anomalous osmosis" through solid membranes if it depends, as many suppose, on the diffusion of electrolytes through pores. We therefore suggest the term "anaphoresis" for the phenomena described here.

Measurements of the mutual solubilities of water, guaiacol, and trichloroacetic acid and of water, guaiacol, and acetone are given and are discussed in relation to the diffusion experiments. To give a complete picture of the process of diffusion we need to know the activities and concentrations in all parts of the system. The difficulties of achieving this are obvious.

The solubility relations are such that a concentration gradient of trichloroacetic acid in guaiacol produces a concentration gradient of water in the same direction, but the activity gradient of water is in the opposite direction.

Since in certain respects guaiacol acts like some protoplasmic surfaces it seems possible that similar phenomena may occur in living cells. If so these results have an obvious bearing on the movement of water in the organism and on methods of studying permeability. It becomes necessary to know to what extent a substance entering or leaving the cell appears to carry water with it in the manner here indicated.

In certain of the diffusion experiments the water takes a circular path, passing out of the dilute solution at one point and back into it (as vapor) at another. This circular path recalls the situation in the kidney where the water continually passes out of the blood into the glomerulus and tubule and then back into the blood from the tubule (where the solution is more concentrated). In both cases the circular path of the water is an essential feature.

ELECTRON BOMBARDMENT OF BIOLOGICAL MATERIALS

II. The Rate of Death of Fungus Spores Bombarded in Vacuum with Cathode Ray Beams from 4 Kv.-15 Kv.

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INTRODUCTION

The profound effects which can be brought about in biological materials when they are subjected to beams of ionizing radiation, and in particular to x-rays, have long been well known. Such phenomena form the extensive basis of work in x-ray cancer therapy, and in the production of mutations by x-rays. It is an equally familiar fact that the total energy which need be absorbed from such a beam by the biological material in order to bring about marked changes within it may be extremely small. Such considerations led Dessauer in 1922 and 1923 (1) to propound his "point-heat" hypothesis, which assumed that the biological action of x-rays was localized to very restricted, discrete points within the irradiated material, where the quantity of energy absorbed per unit volume would indeed be high, although if integrated over the material as a whole it might be very low. Dessauer thought entirely in terms of thermal agitation. Later, as the concepts of ionization under x-ray bombardment became clearer, the idea of discrete energy absorption from the x-ray beam was retained, but the concept of a discontinuous absorption of heat energy in the material was replaced by that of ionization consequent upon the shower of secondary electrons released by the x-ray beam. Crowther (2), Condon and Terrill (3), Holweck and Lacassagne (4), Curie (5), Glocker (6), Wyckoff (7), Mayneord (8), and others have further developed this idea, especially in relation to the concept of the "sensitive volume" for various biological effects. It was further demonstrated by Stadler (9), Wyckoff and Rivers (10), Haskins and Moore (11), and others that high voltage cathode rays are qualitatively identical in their biological effects with x-rays. All of the considerable body of evidence now accumulated indicates clearly that the biological effects of x-rays are due primarily, not to the radiations themselves, but

to the shower of photo- and Compton recoil electrons to which they give rise, and to the ionization which these electrons produce.

For the interpretation of the biological action of x-rays it is therefore of interest to investigate directly the effect of the corresponding electrons upon living cells. Such a procedure appears to have several advantages in the direct quantitative study of x-ray biological effects in general, and of x-ray cancer therapy in particular. The showers of electrons produced in tissue when irradiated with even a monochromatic x-ray beam, are far from homogeneous either in velocity or distribution, due to scattering and energy degradation suffered by the beam, to the combined presence of photo- and recoil electrons, to the wide spread of energies delivered to the recoil electron, and to the slowing down of all the electrons. There will, to be sure, be a maximum velocity for each x-ray wavelength, but the velocity distribution will be a very broad one. With cathode rays, however, it is easily possible to produce beams of a high degree of homogeneity in both energy and distribution.

A careful investigation of the relative mortality of unicellular organisms—and especially of carcinomatic tissue cells—under equivalent dosages of electron irradiation, as a function of electron velocity over a wide range of velocities, might be expected to provide interesting information as to the relative lethal efficiency of x-rays over a correspondingly wide wavelength band. A comparative study of differential killing as between cancerous and non-cancerous tissue would be especially interesting. Again, studies of the variation of survival ratio for single cells under bombardment by cathode rays with varying rates of delivery of identical dosages should yield very useful data in confirming or disproving the validity of the Bunsen-Roscoe reciprocity relationship for x-rays. The method has the advantage that a cathode ray tube has more flexibility for this purpose than semi-standard x-ray equipment. Third, it is possible to so design a cathode ray tube that the electron beam shall penetrate the cells to any desired depth, thus acting as a sort of blunt-ended probe (because of the high release of energy at the end of the electron track) wherewith to determine the relative sensitivity to ionization of various parts of the living single cell. Again, studies of the phenomenon of mutation under ionizing radiation, already observed very many times with x-rays and with high velocity cathode rays, can be investigated much more quantitatively by the use of low velocity, low current electron beams. And lastly, work of this sort can be made directly comparable with studies of the biological effects of proton beams and their corresponding neutrons.

These reasons have prompted the initiation of a research program which

has been partly described elsewhere (12). It envisages a careful study of the biological effects of cathode ray irradiation by beams of electrons at energies ranging from 20 to 150,000 electron volts and current densities from 10^{-7}-10^{-1} amperes per square cm. upon single cells, upon cancer tissues, and upon other biological materials which appear to be of particular interest.

When using cathode rays of energies lower than about 20,000 electron volts, it is necessary that all work shall be done in high vacuum, of the order of 10^{-5} mm. of mercury, in order to provide adequate insulation and to prevent undue scattering of the electron beam. For this energy range, therefore, it has been necessary to select biological objects which are capable of withstanding these low pressures. For this purpose, spores of the ascomycete fungi *Aspergillus niger* and *Penicillium sp.* have proven most suitable. The present paper deals entirely with the effects of cathode ray beams of currents from $1 \times 10^{-7} - 3 \times 10^{-6}$ amperes per square cm. and energies from 4-15 electron kilovolts on such spores, inactivation of the spore being taken as the end-point.

PREVIOUS WORK

A number of investigations of the biological effects of high voltage cathode rays have been made by various workers. Thus Wyckoff (10) investigated their lethal effect upon bacteria, Stadler (9) studied the mutation of various grains under the radiations from a Coolidge high voltage cathode ray tube, and Haskins and Moore (11) studied the inhibition of growth in pollen grains and mold spores when bombarded from a similar source, operated at 250 kvp.

Very much less work, however, has been attempted with low velocity cathode ray beams, comparable in energy with the electronic showers released by x-rays. In 1929, D. A. Wells, at the University of Cincinnati (13), designed a tube to produce such beams, and used them in preliminary studies of the effects of electron beams of high current density and very low voltage on the bacteria *Staphylococcus aureus* and *Bacillus coli*. This work, unfortunately, was discontinued rather shortly after its inception. No further work, so far as is known to the authors, has been attempted in this field.

Source and Character of Cathode Ray Beam

The cathode ray tube designed and used for this work has been more fully described elsewhere (12). It consists essentially of an electron gun, built of glass, designed to produce the cathode beam, connected to a raying box of brass which contains the equip-

ment for holding and manipulating the spores. The electron gun comprises a straight bare-wire tungsten filament mounted vertically, accelerating electrodes, and a pair of deflecting plates, by which the mechanically collimated cathode ray beam is bent at 90° in an electrostatic field, to eliminate any effect of radiant heat upon the irradiated material. After being bent through a 90° arc, the beam from the electron gun is further accelerated and a portion of it passed through a narrow slit (0.351 mm. by 6.31 mm.) with its long axis vertical, into the raying box. Steady D.C. voltage for this acceleration is provided by means of a General Electric kenetron testing set and a capacitor. Within

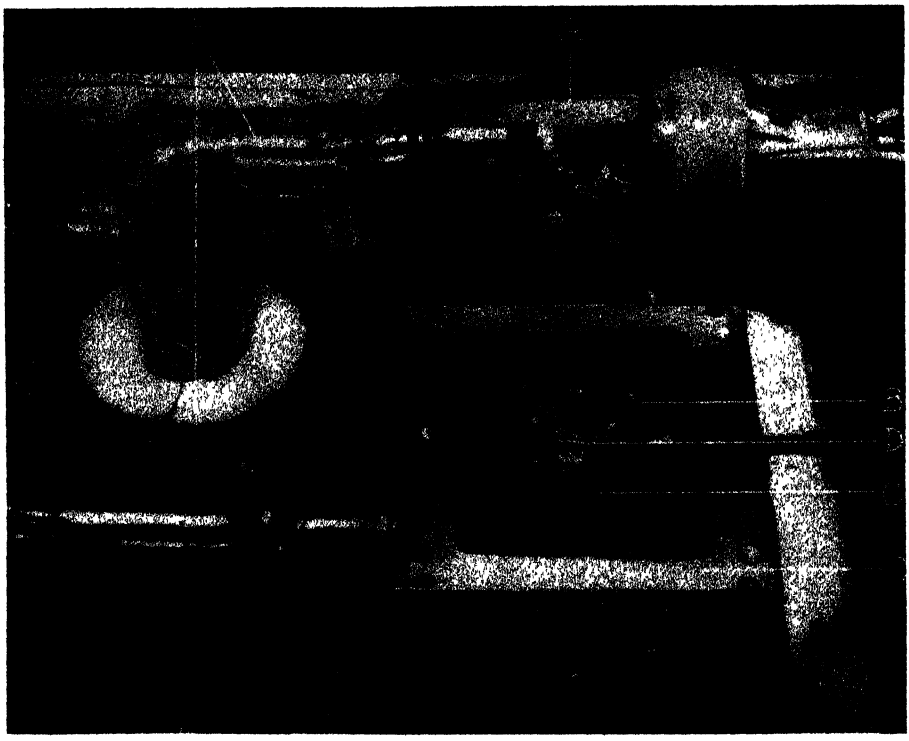

FIG. 1. Raying box with drum in position. A, electron gun; B, rotatable drum; C, specimen slides in clips; D, Faraday cage; E, raying box; F, pump connections.

the raying box is mounted a rotating drum, shafted vertically through a grease-packed vacuum seal in the bottom of the box, and geared to a telechron motor, so that it is possible to rotate it very uniformly. This drum is so mounted relative to the slit system connecting the electron gun and the raying box that it rotates very close to it. On the periphery of the drum are clipped twenty-three small slides, designed to hold the spores to be irradiated. These slides measure 2.5 cm. × 0.5 cm., are of brass, and have a heavy chromium-plated surface, highly polished.

A Faraday cage, connected to a D.C. amplifier for measurement of the electron current, is mounted in the center of the drum, and opens onto the drum surface through a slit large enough to pass the entire beam entering the raying chamber.

As the drum is slowly revolved by the telechron motor, each slide on its periphery, and finally the opening of the Faraday cage, is brought opposite the slit to the electron gun, so that, when a beam is produced, a band of 0.631 cm. width is scanned across the center of each slide. Provision is made for disengaging the shaft of the drum from the telechron motor, so that the opening of the Faraday cage, or any slide, can be brought opposite the slit system at will during a run. A general view of the electron gun and the raying box, with cover removed and drum in position, is shown in Fig. 1.

This tube is capable of providing cathode ray dosages from 6×10^{-9} to 4×10^{-5} coulombs per square cm., at energies of 4 to 20 electron kilovolts, with electron beams which are highly homogeneous in both energy and distribution over the rayed area of the slide.

Materials and Methods

Mature spores of a species of the ascomycete fungus *Penicillium* were at first used in this work. It was found, however, that spores of the black mold *Aspergillus niger* were considerably more satisfactory because of the single, centrally located nucleus, of the uniform size, and, particularly important, that they are less adhesive and liable to clustering. All the work to be described here, therefore, was done with spores of *Aspergillus niger* taken from standardized cultures grown upon a potato-maltose-agar medium under standard conditions. The spores used were all taken from cultures which had been sporulating for at least 3 weeks. Frequent test counts were made to be certain that the percentage of germination in the controls remained consistently high.

The spores were spread, in a single layer, upon the chromium-plated slides already described. Before each irradiation these slides were carefully cleaned and sterilized by immersion in alcohol for at least 30 minutes. When the slides were dry, the spores were spread on them with a soft camel's-hair brush. The slides were then mounted in sterile clips and kept in sterilized Petri dishes until ready for use. In this condition they could safely be transferred from place to place and kept for some time before irradiation with no chance of contamination.

Immediately before irradiation, the spore-bearing slides were transferred to the irradiation drum, and the latter was mounted in position in the raying box of the tube. The system was sealed, vacuum pumps were started, and the tube was evacuated to a pressure of the order of 10^{-5} mm. of mercury. The time required for this procedure was of the order of 75 minutes. The actual irradiation then took place. Each slide was bombarded with a given dosage of electrons at an accurately known voltage according to a predetermined schedule. Normally two or three slides were not irradiated in each run, and were used as controls. After all slides had been irradiated, a procedure which usually took about 20 minutes, the system was allowed to stand under vacuum for 40 to 45 minutes to permit the mercury pumps to cool. Air was then admitted and the irradiation drum removed. Care was taken to admit the air into the vacuum chamber in such a manner as to prevent foreign bodies in the outside air from being swept into the chamber and onto the slides. To check the efficacy of the precautions taken against contamination at this time, blank slides were placed in the position of normally loaded ones and careful microscopic examination was made after a

regular run, but no material has ever been found which had been swept in from the outside nor were spores carried from slide to slide.

The total length of time that the spores were subjected to high vacuum was about 2 hours and 20 minutes. Extensive study of evacuated controls has indicated that spores of *Aspergillus niger* of the age and type irradiated are capable of withstanding longer periods of vacuum than this without detectable effect.

After the drum was removed, the slides were placed again in the sterile clips, preparatory to transferring the irradiated spores to agar culture, or the transfer was made directly. The spores were transferred from the surface of the slide to that of an agar plate in a Petri dish by the simple procedure of laying the slides on the agar surface, leaving them in position for a few seconds, and then removing them. The spores then remained firmly fixed on the agar surface with very little derangement of their initial distribution.

Potato-maltose-agar was used throughout the work. Each run required at least three Petri dishes. A control group consisting of spores which had been subjected to treatment identical with the irradiated ones, except that they had been completely shielded from electrons, was always included in each dish. As a further control, a sowing was made for each run of a group of spores which had not been subjected to vacuum.

Counting of the spores began from 5 to 7 hours after the commencement of incubation, depending upon the end-point of effect being sought; and was completed in from 3 to 4 hours.

Four distinct effects were found, of which only the last will be considered in detail in this paper. At very low voltages a distinct shortening of germination time was observed, which is fully described elsewhere (15). At voltages from 11 to 15 kv., germination became very irregular and uneven at certain dosages and many spores swelled extensively without ever producing germ tubes. Cultures obtained from these spores have shown several mutations. Such mutations have been described elsewhere (14) and additional ones will be further described later. Marked retardation of growth with consequent stunting, was also found. And finally, the effect most generally observed, and fully described here, was that of complete inactivation of the cell. Two criteria can be used for this end-point. A cell invariably swells before the production of a germ tube, and the end-point for "killing" may be taken either as the swelling of a cell without the production of a mycelium, or as failure of the cell to swell, so that it is altogether inert. The form of the curves for both types of data has been found to be closely similar, but the abscissas will differ, since in each culture a number of spores swell but do not produce mycelia. The former criterion has been used for the data presented herein.

In counting spores, the stage of the microscope was fixed so that the field of vision was near the edge of the irradiated band. The stage was then moved so that the field of vision scanned a narrow strip across this irradiated band. Spores were counted as living if they had produced any

sign of a germ tube at the time of counting, otherwise as dead. The number of spores counted for each slide ranged from 250 to 500.

As a precaution to eliminate any possibility of preconceived notions concerning the data, the worker counting a given slide was never informed until long after the count as to the conditions of irradiation of that slide. Even the order of slides during irradiation was often transposed, unknown to the worker who counted the spores from them, so that there was no possibility of any anticipation of results. The data were rearranged and calculated by the worker who had done the irradiation.

Survival ratios were computed for each group of spores cultured, and defined as the quotient of the percentage germination of the sample from the irradiated group divided by that from the control cultured with it. In order to increase the accuracy of the data and eliminate the effect of minor changes in the biological material a weighted average survival ratio from 15 to 20 slides irradiated with the same energy and density of cathode rays was taken in the following manner:

Let

$(L_r)_k$ = number living counted on the kth slide
$(D_r)_k$ = number dead counted on kth slide
$(L_r)_k + (D_r)_k = (T_r)_k$ = total number counted on the kth slide
$(L_c)_k$ = number living counted on the control for the kth slide
$(D_c)_k$ = number dead counted on the control for kth slide
$(L_c)_k + (D_c)_k = (T_c)_k$ = total number counted on the control for the kth slide

Then

$$\left(\frac{L_c}{T_c}\right)_k = \frac{1}{M_k} \quad \text{fraction living on the control for the } k\text{th slide}$$

Then the unweighted average survival ratio will be

$$(S.R.)_{\text{unweighted}} \frac{1}{n} \sum_{k=1}^{n} \left(\frac{L_r}{T_r} \cdot M\right)_k$$

Slides on which a small total count had been made will have just as much effect in the average in this expression as slides with a large total count. An average which will take this fact into account can be easily obtained if the size of the total count be used as weighting factor. The expression for the survival ratio then becomes:

$$(S.R.)_{\text{weighted}} = \frac{\sum_{k=1}^{n} (L_r M)_k}{\sum^{n} (T_r)_k}$$

RESULTS

The expression derived in the preceding section was used to determine the survival ratios of the spores for each point, several slides having been treated with the same dosage in each run. The same schedule, moreover, was repeated seven or eight times over a period of a week or more.

The dosage received by each spore sample was readily computed in the following fashion:

Let

D = the dosage
i = the current through the slit system, in amperes
v = the rate of travel of the slide past the slit = 0.018 cm./sec.
h = the height of the slit 0.631 cm.
e = the charge on the electron, in coulombs
A = the average cross-sectional area of a spore
E = the potential difference used to accelerate the electron beam, in volts
k = the number of ergs in an electron-volt

Then the dosage expressed as the current density in coulombs per square cm. incident on the slide is $D_c = \frac{i}{vh} = 88 \times i$, or the dosage expressed as the total number of electrons striking a single spore is $D_n = \frac{iA}{vhe} = 4.4 \times 10^{13} \times i$. Finally, the dosage expressed as the energy in ergs delivered to each spore is $D_e = \frac{kiA\,E}{vhe} = 7.06 \times i \times E$.

For each voltage the current was so chosen that the full range of survival ratios for the material was covered, this current range having been experimentally determined. The probable errors were computed for each point.

The results, for 3.88, 4.80, 5.80, 6.72, 7.60, 8.6, 11.5, and 14.4 kv. are shown in Figs. 2, 3, 4, and 5, the vertical bars indicating the magnitude of the probable error.

It is of great interest to consider whether the same total quantity of energy applied to a given spore produces the same percentage inactivation regardless of the velocity of the electrons. As a criterion of this, the curve shown in Fig. 6 has been constructed, in which the energy in ergs necessary to reduce the survival ratio to 50 per cent is plotted against voltage. It will be seen that the curve shows a marked dip. It is to be remembered, however, that this curve represents the incident rather than the absorbed energy. The absorbed energy, because of the geometry of the spore, complete penetration by the electrons, and other factors, may not

vary in the same fashion. This point will be further elucidated in a theoretical interpretation of results soon to be published.

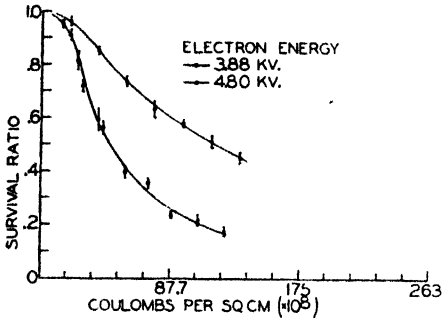

FIG. 2

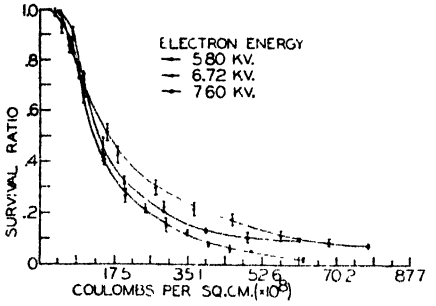

FIG. 3

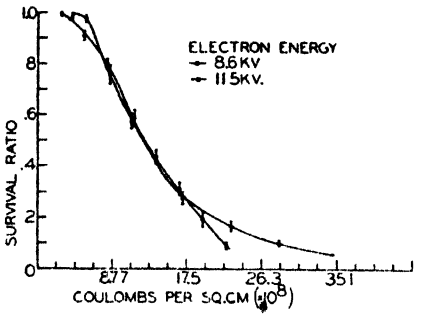

FIG. 4

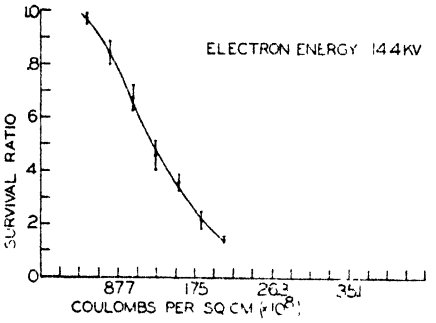

FIG. 5

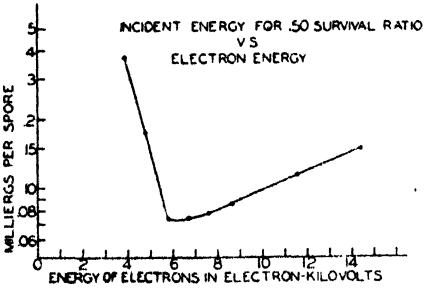

FIG. 6

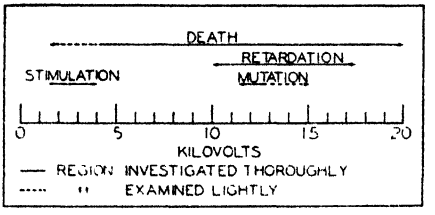

FIG. 7

In Fig. 7 is shown a diagram representing the range of cathode ray velocities which has been investigated, and showing the portion of that range within which each of four described effects was observed. These effects, as has been mentioned, have been or will be described elsewhere.

SUMMARY

A study has been undertaken of the rate of inactivation of spores of the ascomycete fungus *Aspergillus niger* when bombarded in vacuum with homogeneous beams of cathode rays of energies from 4 to 15 electron kv. and current densities of 1×10^{-7} to 3×10^{-6} amperes per square cm. These velocities and densities are in the range of those of showers of secondary electrons produced in biological materials irradiated with moderately soft x-rays, and so may be made to serve as quantitative indicators of the mechanics of x-ray action. Four qualitative effects are described.

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STUDIES ON CELL METABOLISM AND CELL DIVISION

III. Oxygen Consumption and Cell Division of Fertilized Sea Urchin Eggs in the Presence of Respiratory Inhibitors

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Although it has long been recognized that cell division in fertilized sea urchin eggs cannot take place in the absence of oxygen (1, 2) or in the presence of sufficiently high concentrations of respiratory inhibitors like potassium cyanide (2, 3) or phenylurethane (4), with the exception of the case of oxygen lack dealt with by Amberson (5), no simultaneous measurements of rate of oxygen consumption and rate of cell division in the presence of respiratory inhibitors have previously been made.

The experiments reported in the present paper, which were performed in the course of an extensive study of the chemistry of cell division, were made to determine the rate of oxygen consumption of fertilized sea urchin eggs in those concentrations of respiratory inhibitors which just suffice to block cell division. From such data, it was hoped that a decision might be made as to whether or not the adverse effects of a number of reagents on cell division might be due to their effects on the oxygen consumption of the dividing cells. Such data were needed also as a basis for interpretation of the effects of a number of respiratory stimulants on cell division in fertilized *Arbacia* eggs which are to be presented in the next paper of this series.

EXPERIMENTAL METHODS

Measurements of oxygen consumption and cell division were made by the technique previously described (6), using a final egg concentration of 2 per cent by volume. The experiments were conducted during the summers of 1934 to 1939, inclusive, at Woods Hole.

The non-gaseous reagents were dissolved in sea water in a concentration sufficient to give the final concentrations shown in the tables and figures; the solutions were then adjusted to the desired pH, which was that of sea water, 8.0, unless otherwise indicated. The reagents were tipped into the egg suspension from the side arms at the times specified. In later experiments (1937–1939), the final egg suspension in the flasks was made to contain 0.01 M glycylglycine as a buffer to hold the solution at pH 8 during the experiment.

The technique for use of low oxygen pressures and carbon monoxide mixtures was as follows: Previous to the beginning of the experiment mixtures of gases were made up and stored in glass bottles over water, the percentages of oxygen being roughly adjusted. As soon as the eggs were in the flasks and the flasks attached to the manometers, the manometers were attached to a pressure equalizing device which permitted the evacuation of each Warburg manometer and its flask with a Cenco pump without disturbing the manometer fluid; this is essentially the procedure used in filling Barcroft manometers. The pressure was reduced to the vapor pressure of water. By means of a three-way stopcock the desired gas was then admitted and the flask was shaken lightly. This evacuation and refilling was done three times for each flask and gas mixture. At the same time, another set of manometers and flasks was evacuated and refilled with the respective gas mixtures to be analyzed for oxygen by the method of Warburg (7). All this evacuation and refilling required about 20 minutes for a set of twelve manometers and flasks. This meant that the first effective exposure of the eggs to each gas mixture took place at 20 to 30 minutes after fertilization. As soon as this procedure of filling was completed, the vessels were put into the water bath and equilibrated. The solubilities of oxygen, nitrogen, and carbon monoxide in sea water are all very low and therefore no significant change in the gas ratio occurred during equilibration.

The center cup in each flask contained 0.2 cc. 5N sodium hydroxide. In some preliminary cyanide experiments this sodium hydroxide was saturated with potassium cyanide. This procedure is no doubt a valuable one when the cyanide for the physiological experiment is contained in an acid medium. However, for experiments in sea water at pH 8, saturation of the sodium hydroxide by the cyanide proved undesirable because a small amount of the cyanide distilled back into the egg suspension, giving a distortion of the effect of a given initial concentration of cyanide. The opposite effect, distillation of cyanide from the egg suspension into the center cup, was found not to be significant for the present results. This was clearly demonstrated by the fact that the effect of a fixed cyanide concentration (down to 10^{-5}M) was constant with time and showed no decrease as it would if the cyanide were gradually distilling out of the egg suspension.

All experiments were performed at 20°C.

EXPERIMENTAL RESULTS

Low Oxygen Tension.—Oxygen consumption and cell division were measured simultaneously with samples of eggs exposed to oxygen-nitrogen mixtures in which the oxygen content varied from 20 per cent down to 0.1 per cent; in all cases the initial exposure was made at 20–30 minutes after fertilization. Cell division was inhibited slightly in 2 per cent oxygen, more markedly in 1.5 and 1.0 per cent, completely and reversibly in 0.3–0.4 per cent (Fig. 1). Complete inhibition of cell division under these conditions occurred at an oxygen tension which reduced the rate of oxygen consumption to 32 per cent of the control value in air. 50 per cent inhibition of the rate of division was produced at a rate of oxygen consumption which was approximately 50 per cent of that in air. This result confirms and extends the observation of Amberson (5) who found that cell division

in fertilized eggs of *Arbacia punctulata* was slowed when the oxygen consumption was under 80 per cent of that in the control and completely blocked when the oxygen consumption was under 35 per cent of that in the control.

It has been found by Harvey (8), from observation of living eggs, that complete anaerobiosis causes immediate and reversible cessation of development at all mitotic stages in the fertilized sea urchin egg. Studies on fixed and stained eggs have confirmed this result (9). In addition, by a careful analysis[1] of the cytological data, it can be shown that when fertilized *Arbacia* eggs are exposed to a 1 per cent oxygen–99 per cent nitrogen mixture the greatest relative retardation of development occurs at the prophase stage, suggesting that this stage in mitosis may be somewhat more sensitive to oxygen lack than other mitotic stages.

Carbon Monoxide.--Oxygen consumption and cell division were measured simultaneously in mixtures of oxygen and carbon monoxide in which the oxygen content varied from 20 per cent down to 0.3 per cent. With CO mixtures in the dark, cell division was approximately 50 per cent inhibited at 2.6 per cent oxygen, completely and reversibly inhibited at 0.7 per cent oxygen, at which point the rate of oxygen consumption was 32 per cent of that in the control (Fig. 1). The complete cell division block occurred at the same level of oxygen consumption as when simple oxygen lack was the division inhibiting factor; hence carbon monoxide appears to have no effect on division other than that arising from its effect on oxygen consumption. This conclusion is supported by the behavior of the eggs treated with carbon monoxide under strong irradiation from a carbon arc lamp (Table *I*, A).

In a sample of eggs kept in the dark for 220 minutes in a mixture of 1.6 per cent oxygen–98.4 per cent carbon monoxide the oxygen consumption was reduced to about 50 per cent, and the division to about 20 per cent, of the control values.

In a parallel sample of eggs kept in the dark for the first 100 minutes, and then illuminated for the final 120 minutes, the oxygen consumption for the irradiated period was about 90 per cent of that in the control; the division during this irradiated period was also approximately equal to that which would have taken place in the absence of the carbon monoxide, as shown by the following analysis. At 100 minutes the sample of eggs in carbon monoxide in the dark would show approximately 0.5 divisions per egg (*i. e.*, about 50 per cent undivided and about 50 per cent in the two cell

[1] These data will be presented in detail in another publication by H. J. Fry, G. H. A. Clowes, and M. E. Krahl.

stage). If the eggs began dividing again at this point when the light was turned on, and developed thereafter at the normal rate, they would be expected to show approximately 3 divisions per egg at the end of the

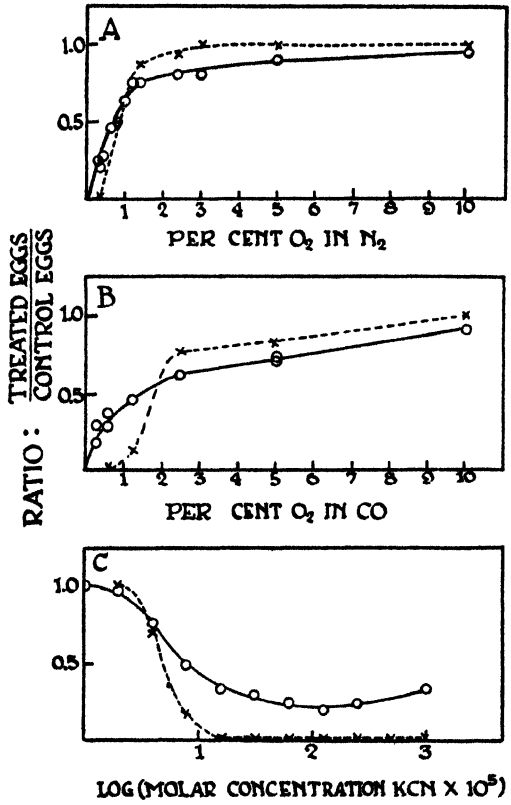

FIG. 1. Oxygen consumption and cell division of fertilized eggs of *Arbacia punctulata* in: *A*, various partial pressures of O_2 in N_2; *B*, various partial pressures of O_2 in CO (in dark); *C*, various concentrations of KCN at pH 8.0. All reagents were added 30 minutes after fertilization. Temperature, 20°C. In Figs. 1–3

O-O $\dfrac{O_2 \text{ consumed in treated eggs}}{O_2 \text{ consumed in control eggs}}$

X-X $\dfrac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$

experiment. They actually showed 2.27 divisions, about 80 per cent of the maximum possible under these conditions, so that exposure to light almost completely reversed the division inhibition by carbon monoxide.

It has been shown by Warburg (10) that the effects of carbon monoxide

on the respiration of yeast can be interpreted by assuming that the carbon monoxide combines with an iron containing pigment of the cell; the extent of this combination is approximately described by the equation:

$$\frac{n}{1-n} \cdot \frac{pco}{po_2} = K, \tag{1}$$

TABLE I

Oxygen Consumption and Cell Division of Fertilized Eggs of Arbacia punctulata in Oxygen-Carbon Monoxide Atmospheres with and without Illumination from a Carbon Arc Lamp

(A) In 1.6 per cent O_2–98.4 per cent CO					(B) In 2.1 per cent O_2–97.9 per cent CO				
Treatment	Period after fertilization	Light	Oxygen consumption	Divisions per egg at 220 min.	Treatment	Period after fertilization	Light	Oxygen consumption	Divisions per egg at 260 min.
	min.		*c.mm. per 10 c.mm. eggs*			*min.*		*c.mm. per 10 c.mm. eggs*	
None	0– 40	Off	Not measured		None	0– 50	Off	Not measured	
	40–100	Off	2.5			50–110*	Off	2.5	
	100–160	On	2.9			125–185	On	2.3	
	160–220	On	2.9	3.94		200–260	Off	2.4	4.62
CO	0– 40	Off	Not measured		CO	0– 50	Off	Not measured	
	40–100	Off	1.9			50–110	Off	1.9	
	100–160	Off	1.8			125–185	Off	1.6	
	160–220	Off	1.3	0.82		200–260	Off	1.3	1.41
CO	0– 40	Off	Not measured		CO	0– 50	Off	Not measured	
	40–100	Off	1.6			50–110	Off	1.8	
	100–160	On	2.3			125–185	On	2.7	
	160–220	On	2.8	2.27		200–260	Off	1.2	2.08

* Two 15 minute periods, for which the illumination was like that for the succeeding 60 minute period in each case, were omitted because of the small but appreciable pressure change resulting from binding or liberation of the CO by the eggs during these periods.

where n is the fraction of the respiration not inhibited, and pco and po_2 are the carbon monoxide and oxygen partial pressures, and K is a constant equal to about 5 for yeast provided with a plentiful supply of glucose as substrate. Runnström (11) found this equation to hold, with $K = 21$, for the effects of carbon monoxide on the oxygen consumption of fertilized eggs of the European sea urchin, *Paracentrotus lividus*. The carbon monoxide data of Fig. 1 conform satisfactorily to an equation of this type with $K = 64$ for the fertilized eggs of *Arbacia punctulata* (Table II).

In 20 per cent oxygen–80 per cent carbon monoxide there was a slight stimulation of oxygen consumption, averaging 20 per cent.

Potassium Cyanide.—Oxygen consumption and cell division were measured simultaneously in samples of eggs exposed, at 20°C., to concentrations of KCN from 10^{-5}M up to 10^{-2}M in sea water at pH 8. Cell division was not inhibited in concentrations of KCN below 2×10^{-5}M; it was 50 per cent inhibited at 5×10^{-5}M, and completely and reversibly inhibited at 1.6×10^{-4}M. At the concentrations of cyanide which produced complete inhibition of division at 20°C., the rate of oxygen consumption was 1.0 c.mm. per hour per 10 c.mm. eggs, or 34 per cent of that in the control (Fig. 1).

TABLE II

Values for K in the Equation $\frac{n}{1-n} \cdot \frac{p_{CO}}{p_{O_2}} = K$ *as Calculated from the Oxygen Consumption of Fertilized Eggs of Arbacia punctulata in Various Mixtures of Oxygen and Carbon Monoxide. Temperature, 20°C.*

Oxygen in carbon monoxide	$\frac{p_{CO}}{p_{O_2}}$	Residual oxygen consumption, n	$\frac{n}{1-n}$	K
per cent		*per cent of control*		
5	19.0	74	2.84	54
3	32.3	66	1.94	63
3	32.3	69	2.22	72
2	49.0	55	1.22	60
2	49.0	55	1.22	60
		Mean value		64

At 24°C. the respective values were 0.8 c.mm. per hour per 10 c.mm. eggs and 16 per cent.

The similarity in the behavior of the eggs with oxygen lack and with CO and KCN inhibition, with all of which the oxygen consumption at the point of division block is 30–40 per cent of that in the control, appears to indicate that the principal effect of KCN on cell division arises from its ability to limit oxygen utilization by these cells and is not attributable to a reducing effect as suggested by Voegtlin (12).

A further point of interest in the data of Fig. 1 is that, within the concentration range in which the egg recovers fully on return to sea water, KCN exerts its maximum inhibiting effect on oxygen consumption of fertilized *Arbacia* eggs at about 1.3×10^{-3}M, a smaller degree of inhibition being produced at higher concentrations (Fig. 1*C*). The minimum residual respiration at 20°C. was approximately 20 to 30 per cent of the control value, varying somewhat with the sample of eggs used. Korr (13) also

observed that these eggs had an appreciable residual respiration in 2.5 × 10^{-3}M KCN. The fact that the residual respiration observed by Korr was somewhat larger than that here reported may possibly be attributable to his use of a cyanide concentration which was, as indicated by the data of Fig. 3 of the present paper, too large to give maximum respiratory inhibition.

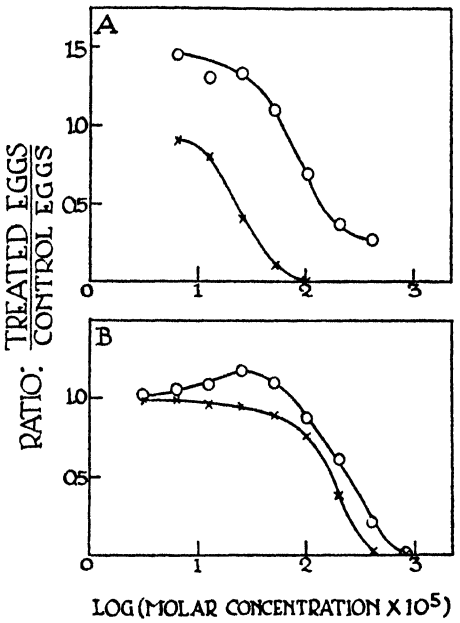

FIG. 2

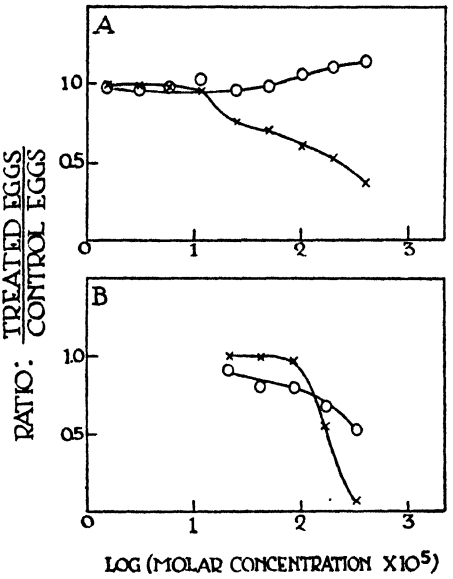

FIG. 3

FIG. 2. Oxygen consumption and cell division of fertilized eggs of *Arbacia punctulata* in: *A*, various concentrations of phenylurethane at pH 8.0; *B*, various concentrations of 5-isoamyl-5-ethyl barbituric acid at pH 7.8. The reagents were added 25 minutes after fertilization. Temperature, 20°C.

FIG. 3. Oxygen consumption and cell division of fertilized eggs of *Arbacia punctulata* in: *A*, various concentrations of γ-(2-methylpiperidyl)-propyl benzoate at approximately pH 6.5; *B*, various concentrations of iodoacetic acid at approximately pH 6.0. The reagents were added 25 minutes after fertilization. Temperature, 20°C.

A residual respiration equal to about 15 per cent of the control value was observed at 24°C. Barron and Hamburger (14) reported complete inhibition of oxygen consumption of fertilized *Arbacia* eggs in 10^{-3} to 10^{-2}M cyanide at 24°C. but did not state whether or not the eggs recovered from the treatment.

Anesthetics Inhibiting Cell Division.—For comparison with the effects obtained with low oxygen tension, carbon monoxide, and cyanide, several

series of comparable experiments were made with representative anesthetic agents.

Of these, the most effective agent for inhibiting cell division was phenylurethane. This substance produced a 50 per cent inhibition of cell division at about 3×10^{-4}M, at which point the oxygen consumption was 130 per cent of the control value. Complete and reversible inhibition of cell division occurred at about 1×10^{-3}M, at which point the oxygen consumption was 70 per cent of that in the control (Fig. 2).

A second anesthetic was 5-isoamyl-5-ethyl barbituric acid, a substance used as a hypnotic for mammals. This agent produced a 50 per cent inhibition of cell division at 1.8×10^{-3}M, with the oxygen consumption at 75 per cent of the control value, and complete inhibition at about 2.5×10^{-3}M, with the oxygen consumption at 20 per cent of the control (Fig. 2).

A third anesthetic was γ-(2-methylpiperidyl)-propyl benzoate, a substance used as a local anesthetic for mammals. This agent produced 50 per cent inhibition of cell division at 2×10^{-3}M and a maximum of 64 per cent at 4×10^{-3}M, the concentration giving a saturated solution in sea water. At these concentrations, the oxygen consumption was not inhibited, the rates being 108 and 106 per cent of the control value, respectively (Fig. 3).

Iodoacetic Acid.—This inhibitor was used because of its potential effects on the early stages of the respiratory process. It must be used in a relatively acid solution in order to insure its penetration into fertilized sea urchin eggs. It produced a 50 per cent inhibition of division at about 1.7×10^{-3}M and a 90 per cent inhibition at about 3.4×10^{-3}M. At these concentrations the rates of oxygen consumption were 67 per cent and 54 per cent of the control value, respectively (Fig. 3).

DISCUSSION

The principal point established by these experiments is that when fertilized *Arbacia* eggs are exposed, 30 minutes after fertilization at 20°C., to low oxygen tension, carbon monoxide, or potassium cyanide, the block to cell division occurs at approximately the same level of oxygen consumption, which is 30 to 40 per cent of that in the control. It has been found (15) that fertilized eggs of *Arbacia punctulata* have a small or negligible rate of glycolysis, both aerobic and anaerobic, so that these eggs depend almost exclusively on oxygen consumption for the energy required to support division. Taken in conjunction with the present experiments, this appears to indicate that, at 20°C., the fraction of the oxygen consumption required to support mitotic processes is at most about 30 per cent of the total; it is probably much smaller than this, as indicated by the fact

that cell division at 24°C. was not completely blocked until the oxygen consumption was reduced to 16 per cent of the control value.

The second point established by the present experiments concerns the nature of the respiratory enzymes in fertilized sea urchin eggs. The sensitivity of the cell division to carbon monoxide and cyanide appears, at first sight, to indicate that mitotic processes in fertilized eggs of *Arbacia punctulata* are dependent on the functioning of a typical cytochrome-cytochrome oxidase respiratory system such as that found in muscle or yeast cells. On closer examination of the data, this idea is not substantiated.

In the first place, repeated examinations of both fertilized and unfertilized eggs of *Arbacia punctulata* (treated with sodium hydrosulfite to reduce both echinochrome and cytochrome) have failed to reveal the presence of cytochrome absorption bands in the eggs; *Arbacia* sperm display a pronounced absorption band at about 600-605 mμ corresponding to the band of cytochrome a. Although these experiments on eggs do not exclude the possibility that cytochrome may be present in relatively small amounts, they indicate that the cytochrome content of the eggs is very much smaller than that of other cells having a comparable rate of respiration sensitive to cyanide and carbon monoxide.

In the second place, the inhibition constant (equation 1, above) for the effect of carbon monoxide on oxygen consumption of fertilized eggs of *Arbacia*, which has a value of 64, is much larger than the corresponding constants for the cytochrome-cytochrome oxidase systems of yeast and muscle (16) which have values from 5 to 10. Even if, reasoning from the results for cyanide, it is assumed that 20 per cent of the egg respiration is insensitive to CO, the value of K is 43; for an egg respiration 30 per cent insensitive to CO, the value of K is 32.

In spite of these two facts which militate against the presence of a typical cytochrome-cytochrome oxidase system in the eggs, there are two further lines of evidence to indicate that the cyanide and carbon monoxide sensitive system of the eggs contains iron.

1. Potassium dithio-oxalate (10^{-2}M), diphenylthiocarbazone (10^{-4}M), and isonitrosoacetophenone (2×10^{-3}M), three reagents which combine with copper, were completely ineffective as division inhibitors, indicating that the adverse division effects of potassium cyanide are not attributable to cyanide-copper reactions.

2. Ball and Meyerhof (17) observed a strong hemochromogen absorption spectrum in fertilized eggs of *Arbacia* treated with pyridine, indicating the presence of a considerable amount of hemin iron.

Taken as a group, these experiments indicate that: (*a*) One or more iron

containing respiratory catalysts are involved in oxygen uptake by *Arbacia* eggs. (*b*) Little or none of the hemin iron of the egg is normally bound to those nitrogenous cellular components which would enable it to appear as cytochrome. (*c*) Such hemin iron as functions as a respiratory catalyst in the eggs is therefore probably bound to one or more cellular components, other than the proteins of cytochrome, which raise the oxidation-reduction potential of the hemin to a point where it can function as a respiratory catalyst, but do not yield a hemin compound with an absorption spectrum powerful enough to be detected in the eggs.

The third point established by the present experimental data is that the effects on cell division of a non-ionizable general anesthetic, phenylurethane, as well as those of an ionizable local anesthetic which produced its intracellular effects on division as the cation, γ-(2-methylpiperidyl)-propyl benzoate, are not attributable to the effects of these agents on the overall respiration of the egg, since division is completely blocked at concentrations of the reagents which reduce the oxygen consumption to a level equal to or only slightly below that of the control.

SUMMARY

1. The effects of a number of respiratory inhibiting agents on the cell division of fertilized eggs of *Arbacia punctulata* have been determined. For eggs initially exposed to the reagents at 30 minutes after fertilization at 20°C., the levels of oxygen consumption prevailing in the minimum concentrations of reagents which produced complete cleavage block were (as percentages of the control): In 0.4 per cent O_2–99.6 per cent N_2, 32; in 0.7 per cent O_2–99.3 per cent CO, 32; in 1.6×10^{-4}M potassium cyanide, 34; in 1×10^{-3}M phenylurethane, 70; in 4×10^{-3}M 5-isoamyl-5-ethyl barbituric acid, 20; in 3×10^{-4}M iodoacetic acid, 53.

2. The carbon monoxide inhibition of oxygen consumption and cell division was reversed by light. The percentage inhibition of oxygen consumption by carbon monoxide in the dark is described by the usual mass action equation with K, the inhibition constant, equal to approximately 60, as compared to values of 5 to 10 for yeast and muscle. In 20 per cent O_2–80 per cent CO in the dark there was a slight stimulation of oxygen consumption, averaging 20 per cent.

3. Spectroscopic examination of fertilized and unfertilized *Arbacia* eggs reduced by hydrosulfite revealed no cytochrome bands. The thickness and density of the egg suspension was such as to indicate that, if cytochrome is present at all, the amount in *Arbacia* eggs is extremely small as compared to that in other tissues having a comparable rate of oxygen consumption.

4. Three reagents poisoning copper catalyses, potassium dithio-oxalate (10^{-2}M), diphenylthiocarbazone (10^{-4}M), and isonitrosoacetophenone (2×10^{-3}M) produced no inhibition of division of fertilized *Arbacia* eggs.

5. These results indicate that the respiratory processes required to support division in the *Arbacia* egg may perhaps differ in certain essential steps from the principal respiratory processes in yeast and muscle.

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STUDIES ON CELL METABOLISM AND CELL DIVISION

IV. Combined Action of Substituted Phenols, Cyanide, Carbon Monoxide, and Other Respiratory Inhibitors on Respiration and Cell Division

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In previous papers of this series it has been shown that certain substituted phenols produce a large stimulation of the oxygen consumption and a reversible block to the cell division of marine eggs (1, 2). These results have been confirmed and extended to other eggs by Tyler and Horowitz (4), and Dawson (5). As yet there is no complete understanding of the mechanism of this respiratory stimulation and almost no knowledge of the manner in which the respiratory and division effects of the substituted phenols are related.

The experiments reported in this paper were made to determine: (*a*) the sensitivity of the substituted phenol stimulated respiration to various types ot respiratory inhibitors, and (*b*) the possible relationship between the respiratory effects and the division inhibiting effects of the substituted phenols.

The experiments were made with fertilized eggs of the sea urchin, *Arbacia punctulata.*

EXPERIMENTAL METHODS

The measurements of oxygen consumption were made by the direct method of Warburg (6) using the technique previously described (2, 7).

The non-gaseous reagents were dissolved in sea water in a concentration sufficient to give the final concentrations shown in the tables and figures, and then adjusted to the desired pH, which was that of sea water, 8.0, unless otherwise indicated. 0.5 cc. of each of the solutions was placed in the side arm of the Warburg vessel and tipped into the egg suspension at the time indicated. If two reagents were to be added at different times, the first was added directly to the egg suspension before attaching the flasks to the manometers and the second substance was tipped in during the course of the experiments.

The unfertilized eggs of the sea urchin, *Arbacia punctulata*, were shed in the usual way, washed twice with sea water, fertilized in a large volume of sea water, and allowed to settle. They were then adjusted to a volume which would give a final egg concen-

tration of approximately 2 per cent. In the iodoacetic acid experiments, at 5 minutes after fertilization, the normal sea water was replaced with carbonate free sea water at pH 6.

In each case the egg suspension was transferred to the Warburg flasks by means of a volumetric pipette with a wide opening. The volume and concentration of the egg suspension were adjusted to give a total volume of 5.5 cc. of 2 per cent egg suspension in each flask.

At the end of each experiment, a sample of eggs from each flask was fixed in 0.2 per cent formol; from these samples the extent of division was estimated and expressed as previously described (2).

All experiments were run at 20°C.

EXPERIMENTAL RESULTS

Low Oxygen Tension.—The effects of a series of concentrations of 4,6-dinitro-*o*-cresol on oxygen consumption and cell division of fertilized *Arbacia* eggs were determined at each of several partial pressures of oxygen in nitrogen (Fig. 1). There are three points of interest brought out by these results.

First, respiratory stimulation by 4,6-dinitro-*o*-cresol decreased and finally disappeared as the oxygen tension was progressively lowered.

Second, the degree of inhibition of cell division by each concentration of 4,6-dinitro-*o*-cresol increased as the oxygen tension was lowered. This result supports the conclusion previously reached (2), that the division inhibition produced by the 4,6-dinitro-*o*-cresol is not the result of overstimulation of oxidation by this reagent.

Third, when the oxygen consumption, reduced by exposure of the eggs to 2 per cent oxygen, was raised to the normal level in air by using 4,6-dinitro-*o*-cresol with the 2 per cent oxygen (Fig. 1, IC), there was no corresponding reversal of the inhibition of division associated with this low oxygen tension. On the contrary, the combination of 4,6-dinitro-*o*-cresol with low oxygen tension produced an additive inhibition of division.

Carbon Monoxide.—The effects of a series of concentrations of 4,6-dinitro-*o*-cresol were determined at each of two partial pressures of oxygen in carbon monoxide (Fig. 1). It is particularly interesting to note: (*a*) that at 4,6-dinitro-*o*-cresol concentrations of 10^{-6}M, 2×10^{-6}M, and 4×10^{-6}M with 6 per cent oxygen–94 per cent carbon monoxide, the absolute rate of oxygen consumption was less than that in the presence of the carbon monoxide control, and (*b*) that the percentage reduction of 4,6-dinitro-*o*-cresol stimulated respiration produced by including the carbon monoxide was only 51 per cent in the presence of 3.2×10^{-6}M 4,6-dinitro-*o*-cresol and 66 per cent at 3×10^{-6}M 4,6-dinitro-*o*-cresol (Table I), although the rates of

oxygen consumption were the same for the two concentrations of this reagent when the carbon monoxide was absent.

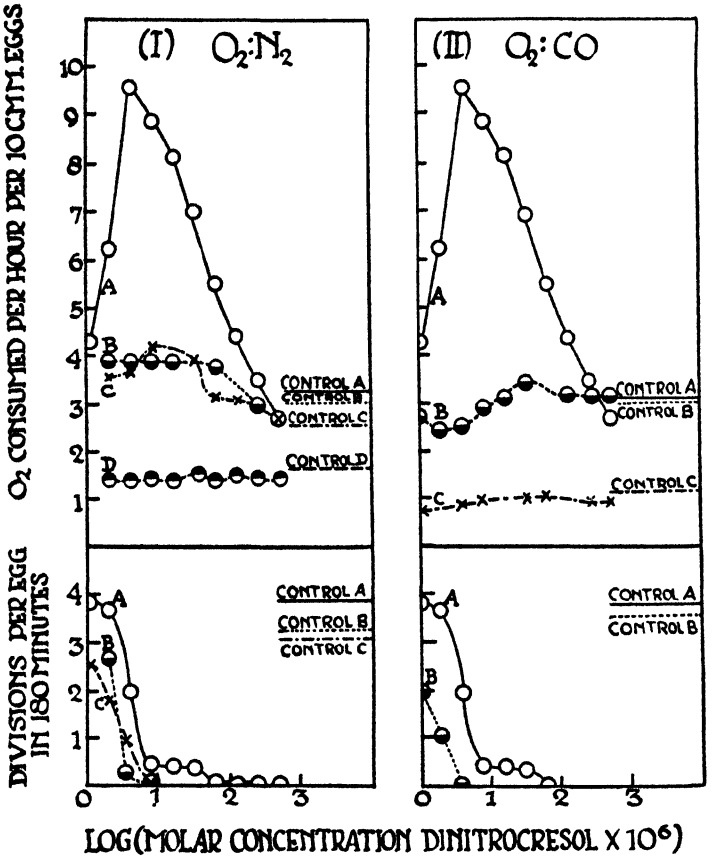

FIG. 1. Effect of various concentrations of 4,6-dinitro-*o*-cresol on oxygen consumption and cell division of fertilized *Arbacia* eggs: I*A* and II*A*, in air; I*B*, in 3 per cent O_2-97 per cent N_2, I*C*, 2 per cent O_2-98 per cent N_2; I*D*, in 0.5 per cent O_2-99 5 per cent N_2; II*B*, in 6 per cent O_2-94 per cent CO; II*C*, in 1.5 per cent O_2-98.5 per cent CO.

In Figs. 1–4 the horizontal lines marked control *A*, etc., are the control levels of oxygen consumption, or division, for the similarly marked curves. Where a division curve corresponding to an oxidation curve is not given, all cleavage was stopped.

All reagents were added 20–30 minutes after fertilization. Temperature, 20°C.

This result would be accounted for if, in greater than optimum concentrations of 4,6-dinitro-*o*-cresol, part of the oxygen consumption passed through carbon monoxide insensitive respiratory catalysts; it would also be explained if high concentrations of substituted phenol produced a decrease in the affinity of the respiratory catalytic system for carbon monoxide.

From the results with cyanide given below, this latter explanation appears to be the correct one.

In 1.5 per cent oxygen–98.5 per cent carbon monoxide, which reduced the oxygen consumption of the eggs to 36 per cent of the control value, the oxygen consumption was not stimulated by any concentration of 4,6-dinitro-*o*-cresol up to 5.12×10^{-4}M.

TABLE I

Percentage Reduction of 4,6-Dinitro-o-Cresol Stimulated Respiration by Respiratory Inhibitors at Various Levels of Oxygen Consumption on the Rising (R) and Falling (F) Segments of the Stimulation Curve

Oxygen consumption	Concentration 4,6-dinitro-*o*-cresol	Segment of curves used	Percentage reduction of stimulated respiration in various reagents					
			2 per cent O_2, 98 per cent N_2	6 per cent O_2, 94 per cent CO	8×10^{-5} M KCN at pH 7.5	10^{-3} M Phenylurethane at pH 7.5	10^{-3} M 5-Isoamyl-5-ethyl barbituric acid at pH 7.5	10^{-3} M Malonic acid at pH 7.5
c.mm. per 10 c.mm. eggs	*moles per l. × 10⁶*							
3.2	0		22	5	59	29	5	5
5.0	1.4	R	30	50	78	44	20	0
	83.	F	38	34	52	27	0	10
6.0	1.8	R	42	60	80	48	8	0
	51.	F	38	45	60	30	7	11
7.0	2.3	R	49	66	83	54	10	10
	32.	F	44	51	68	26	13	20
8.0	3.0	R	56	70	84	60	3	18
	16.	F	49	61	83	33	18	21
9.0	3.9	R	60	72	86	63	10	23
	6.8	F	53	66	86	56	16	23

As in the experiments with low oxygen tension, prevention of 4,6-dinitro-*o*-cresol respiratory stimulation by carbon monoxide produced no reversal of the 4,6-dinitro-*o*-cresol division block; on the contrary, the division inhibiting effects of the two treatments were additive.

As shown in a previous paper (7) the inhibition of oxygen consumption by carbon monoxide in fertilized eggs of *Arbacia punctulata* was almost completely reversed by light. Likewise, the carbon monoxide block to respiratory stimulation by 4,6-dinitro-*o*-cresol, and the additive effect of

the carbon monoxide on cell division were in large part reversed by light of sufficient intensity (Table II), the respiration and cell division being then approximately the same as if the carbon monoxide were replaced by nitrogen. Bodine and Boell (8) have observed that the carbon monoxide inhibition of respiratory stimulation by 2,4-dinitrophenol in grasshopper embryos is reversed by light.

Potassium Cyanide.—The effects of each of ten concentrations of 4,6-dinitro-*o*-cresol were determined at each of five concentrations of potassium

TABLE II

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) and Cell Division of Fertilized Eggs of Arbacia punctulata in 2.1 Per Cent Oxygen–97.9 Per Cent Carbon Monoxide with and without 4 × 10^{-6}M 4,6-Dinitro-o-Cresol. The Eggs Were First Exposed to Reagents 25 Minutes after Fertilization. Temperature, 20°C.

Treatment	50–110 min. after fertilization		125–185 min. after fertilization		200–260 min. after fertilization		Divisions per egg at 260 min.
	Light*	O_2	Light	O_2	Light	O_2	
None-control	Off	2.7	Off	2.4	Off	2.4	4.77
None-control	Off	2.5	On	2.3	Off	2.4	4.62
CO	Off	1.9	Off	1.6	Off	1.3	1.41
CO	Off	1.8	On	2.7	Off	1.2	2.08
4,6-Dinitro-*o*-cresol	Off	6.2	Off	5.6	Off	5.2	3.01
4,6-Dinitro-*o*-cresol	Off	6.2	On	5.2	Off	5.3	3.16
CO and 4,6-dinitro-*o*-cresol	Off	1.9	Off	1.7	Off	1.2	0.03
CO and 4,6-dinitro-*o*-cresol	Off	2.2	On	4.0	Off	1.9	0.95

* All samples were kept in the dark for the first 50 minutes after fertilization. During the 110–125 minute and 185–200 minute periods the condition of illumination for each period was the same as for the immediately following period.

cyanide (Table III). Both the absolute and relative reduction of respiration produced by each concentration of cyanide depended on the concentration of 4,6-dinitro-*o*-cresol simultaneously employed. In concentrations of 4,6-dinitro-*o*-cresol smaller than that producing the optimum respiratory stimulation without cyanide, the percentage reduction by cyanide was invariably greater than in a concentration of the substituted phenol larger than the respiratory optimum, even if the absolute rates of oxygen consumption in the two respective concentrations of 4,6-dinitro-*o*-cresol were the same in the absence of cyanide. For example (Table I), at points of equal oxygen consumption on the ascending and descending segments of the curve of respiratory stimulation by 4,6-dinitro-*o*-cresol, at which the oxygen consumption was 7.0 c.mm. per hour per 10 c.mm. eggs, 8 × 10^{-5}M KCN

produced an 83 per cent reduction on the rising segment of the curve and only a 68 per cent reduction on the falling segment.

Similar experiments were performed with 2,4,5-trichlorophenol as the stimulating agent. As in the case of 4,6-dinitro-*o*-cresol, the addition of cyanide, at points of equal oxygen consumption on the rising and falling segments of the curve, produced a greater reduction on the rising than on the descending segment.

The increase in the optimum stimulating concentration of 4,6-dinitro-*o*-cresol (Figs. 1 and 2, Table III) or 2,4,5-trichlorophenol (Table IV) with increasing concentrations of cyanide or carbon monoxide deserves comment. At first sight, this appears to indicate that the substituted phenols, in high concentrations, stimulate a cyanide insensitive form of respiration. This, however, cannot be the case, since the absolute level of residual oxygen consumption under maximum cyanide inhibition was nearly the same at concentrations of 4,6-dinitro-*o*-cresol (Table V) or of 2,4,5-trichlorophenol (Table VI) less than or greater than the concentration producing optimum stimulation in air. The least unsatisfactory explanation of the data therefore appears to be that, in the higher range of 4,6-dinitro-*o*-cresol concentrations, the affinity of the respiratory system of the fertilized *Arbacia* egg for cyanide or carbon monoxide is less than that in lower concentrations of the substituted phenol. This is apparently not due to competition of the substituted phenol and the cyanide for the iron component of the enzyme system because 4,6-dinitro-*o*-cresol has, in cell free systems, no inhibiting effect on the activity of the respiratory enzymes poisoned by cyanide. The cyanide sensitive systems upon which the 4,6-dinitro-*o*-cresol, in concentrations up to 1000 times the physiological concentrations, had no effect are: indophenol oxidase, catalase, polyphenol oxidase, hemin, pyridine and nicotine hemochromogens, iron and copper as catalysts for cysteine oxidation, and iron and copper as catalysts for ascorbic acid oxidation (9).

Part of the division inhibition produced by 2×10^{-5}M KCN was reversed by either 4,6-dinitro-*o*-cresol or 2,4,5-trichlorophenol (Tables III and IV). The 1×10^{-6}M 4,6-dinitro-*o*-cresol, for example, raised the oxygen consumption from 2.1 to 2.7 (*i.e.* from 68 to 87 per cent of the untreated control) and the division from 2.56 to 3.25 (*i.e.* from 69 to 88 per cent of the untreated control). Repetition of this type of experiment during the seasons of 1936 and 1939 has shown that the reversal of the cyanide division inhibition by substituted phenols can be obtained only when the division in the cyanide alone is about 65–75 per cent of that in the control and when suboptimum respiratory concentrations of the substituted phenol are used.

TABLE III

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) and Cell Division (Divisions Per Egg in 180 Minutes) of Fertilized Eggs of Arbacia punctulata in Various Combined Concentrations of Potassium Cyanide and 4,6-Dinitro-o-Cresol. Both Reagents Were Added 30 Minutes after Fertilization. The pH Was 8.0. Temperature, 20°C.

Concentration of 4,6-dinitro-o-cresol	No KCN		1×10^{-5} M KCN		2×10^{-5} M KCN		4×10^{-5} M KCN		8×10^{-5} M KCN		1.6×10^{-4} M KCN	
	O_2	Division	O_2	Division	O_2	Division	O_2	Division	O_2	Division	O_2	Division
moles per l. × 10⁶												
0	3.2	3.79	2.7	3.23	2.1	2.56	1.9	0.37	1.3	0	1.1	0
1	4.5	3.81	3.4	3.14	2.7	3.25	1.8	0.33	1.1	0	1.0	0
2	6.0	3.66	3.3	1.81	2.2	3.32	2.1	0.11	—		1.2	0
4	9.4	1.97	3.8	0.97	2.4	0.24	2.3	0	1.4	0	1.4	0
8	8.3	0.43	4.0	0.09	2.7	0	2.6	0	1.4	0	1.3	0
16	7.8	0.45	4.4	0.10	3.4	0	2.8	0	1.5	0	1.4	0
32	6.6	0.38	4.3	0.10	3.2	0	2.9	0	2.3	0	1.8	0
64	5.6	0.10	4.2	0	3.0	0	3.0	0	2.3	0	1.7	0
128	4.8	0	3.6	0	2.5	0	2.5	0	2.1	0	1.8	0
256	3.8	0	2.7	0	2.3	0	2.9	0	2.4	0	1.5	0
512	3.0	0	2.7	0	1.9	0	2.6	0	2.1	0	1.8	0

TABLE IV

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) and Cell Division (Divisions Per Egg in 180 Minutes) of Fertilized Eggs of Arbacia punctulata in Various Combined Concentrations of Potassium Cyanide and 2,4,5-Trichlorophenol. Both Reagents Were Added 30 Minutes after Fertilization. The pH Was 7.5. Temperature, 20°C.

Concentration of 2,4,5-trichlorophenol	No KCN		2×10^{-5} M KCN		1.6×10^{-4} M KCN	
	O_2	Division	O_2	Division	O_2	Division
moles per l. × 10⁶						
0	3.1	3.48	2.5	2.36	0.9	0
1	3.5	3.52	2.5	2.93	0.7	0
2	3.2	3.47	2.5	2.77	0.9	0
4	3.9	3.37	2.5	1.85	1.1	0
8	4.3	2.93	2.8	1.53	1.1	0
16	7.5	0.75	2.8	0.70	1.1	0
32	9.2	0.14	3.5	0	1.4	0
64	4.1	0	3.2	0	1.7	0
128	3.0	0	2.4	0	1.6	0
256	2.3	0	2.7	0	1.8	0
512	2.4	0	2.5	0	1.8	0

These two groups of experiments, taken in conjunction with those on the joint effects of 4,6-dinitro-*o*-cresol and carbon monoxide, appear to afford

TABLE V

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) of Fertilized Eggs of Arbacia punctulata in 4,6-Dinitro-o-Cresol with and without Various Concentrations of Potassium Cyanide. The pH Was 8.0. Temperature, 20°C.

Concentration 4,6-dinitro-*o*-cresol	Concentration KCN	O_2
moles per l. × 10^6	*moles per l.* × 10^6	
0	0	3.2
0	32	0.9
0	64	0.9
0	128	1.4
2	0	4.1
2	32	0.9
2	64	0.9
2	128	0.8
128	0	6.1
128	32	1.4
128	64	1.1
128	128	0.8

TABLE VI

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) of Fertilized Eggs of Arbacia punctulata in 2,4,5-Trichlorophenol with and without Various Concentrations of Potassium Cyanide. The pH Was 8.0. Temperature, 20°C.

Concentration 2,4,5-trichlorophenol	Concentration KCN	O_2
moles per l. × 10^6	*moles per l.* × 10^6	
0	0	3.4
0	32	0.5
0	64	0.5
0	128	0.6
10	0	6.3
10	32	0.4
10	64	0.3
10	128	0.4
50	0	3.0
50	32	0.7
50	64	0.7
50	128	0.4

an important clue to the systems involved in respiratory stimulation and division inhibition by the substituted phenols.

In concentrations below the optimum, the respiratory increase produced by the substituted phenols is completely cyanide and carbon monoxide sensitive. As the concentration of the substituted phenol is progressively increased beyond the optimum, these reagents themselves inhibit more and more of the cyanide and carbon monoxide sensitive respiration so that the percentage inhibition by cyanide or carbon monoxide again approaches that in eggs not treated with the substituted phenol. This result appears to be very significant for the interpretation of the effects of the phenols on cell division. It has been shown in previous papers (2, 3) that the minimum concentrations of substituted phenols required to inhibit division completely are invariably the concentrations just larger than those which produce optimum respiratory stimulation. Hence it appears that the substituted phenols begin to inhibit division in those concentrations which produce the initial inhibition of the cyanide sensitive oxidative systems. This suggests that inhibition of cell division by the substituted phenols is to be associated not with over-stimulation of oxidative processes but with the inhibition of a particular type of oxidative process, even though the total oxygen uptake of the eggs may be greatly in excess of the control value. This tentative conclusion is given further support by the fact that the division effects of *greater than optimum* concentrations of the substituted phenols are always additive and never antagonistic to those of cyanide or carbon monoxide.

Phenylurethane, 5-Isoamyl-5-Ethyl Barbituric Acid, and Malonic Acid.—These three agents produce a marked inhibition of the activity of several dehydrogenases (11) but, in contrast to cyanide and carbon monoxide, have little or no effect on the iron containing respiratory pigments (12).

As pointed out by Warburg (13) and emphasized in a previous paper of this series (7), phenylurethane produced, at 10^{-3}M, a reversible block to the cell division of fertilized sea urchin eggs. At this concentration, which reduced the oxygen consumption of the eggs untreated with substituted phenol to 71 per cent of the control value, this reagent prevented maximum respiratory stimulation by 4,6-dinitro-*o*-cresol but was much less effective in this respect than cyanide or carbon monoxide in concentrations giving the same degree of inhibition of respiration in a series untreated with substituted phenol (Fig. 2 and Table I). The inhibition of division by phenylurethane was not antagonized by any concentration of 4,6-dinitro-*o*-cresol.

In a concentration of 10^{-3}M, which produced little or no reduction of respiration in a control series, 5-isoamyl-5-ethyl barbituric acid produced a very slight reduction of respiratory stimulation by concentrations of 4,6-dinitro-*o*-cresol less than that required for the respiratory optimum and

a much larger reduction of respiratory stimulation by concentrations of 4,6-dinitro-*o*-cresol greater than the respiratory optimum (Fig. 3 and Table I). This is the reverse of the picture obtained when cyanide or carbon monoxide were used as inhibitors. At 2 × 10^{-3}M 5-isoamyl-5-

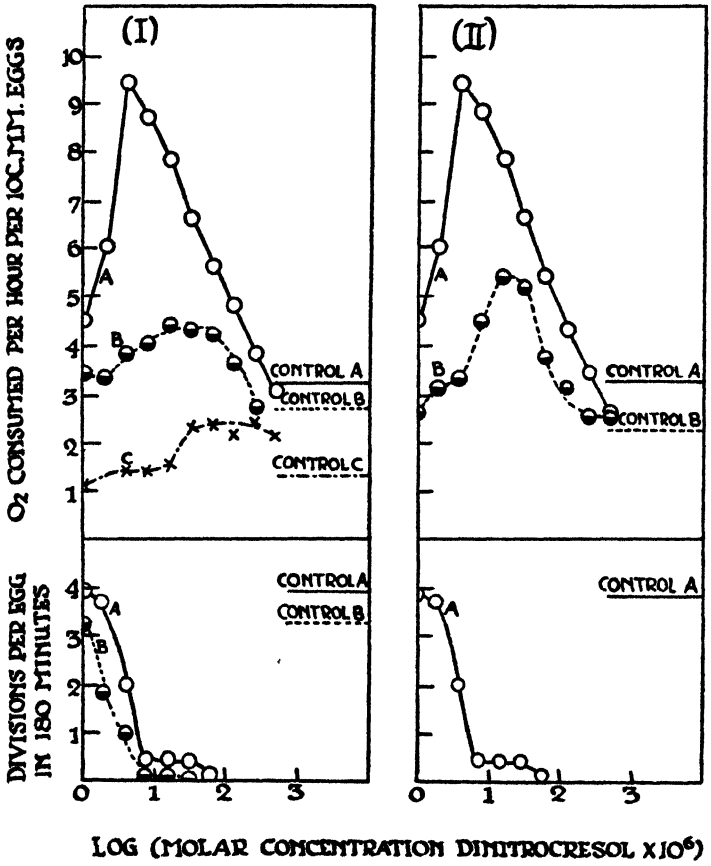

FIG. 2. Effect of various concentrations of 4,6-dinitro-*o*-cresol on oxygen consumption and cell division of *Arbacia* eggs: I*A*, with no other reagent; I*B*, in 10^{-5}M KCN; I*C*, in 8 × 10^{-5}M KCN; II*A*, with no other reagent; II*B*, in 10^{-3}M phenylurethane.

The reagents were added 30 minutes after fertilization. Temperature, 20°C.

ethyl barbituric acid, which reduced the respiration of the untreated eggs to 77 per cent of the control, all respiratory stimulation by 4,6-dinitro-*o*-cresol was prevented. This agent is therefore more efficient than cyanide as an inhibitor of respiratory stimulus by substituted phenols when comparisons are made at concentrations of the two agents which produce equal effects on the respiration of eggs untreated with 4,6-dinitro-*o*-cresol.

The results with malonic acid (Fig. 3 and Table I) were comparable, both in kind and degree, to those obtained with 5-isoamyl-5-ethyl barbituric acid, which contains a substituted malonic acid as part of its structure. At the concentrations used, neither 5-isoamyl-5-ethyl barbituric acid nor malonic

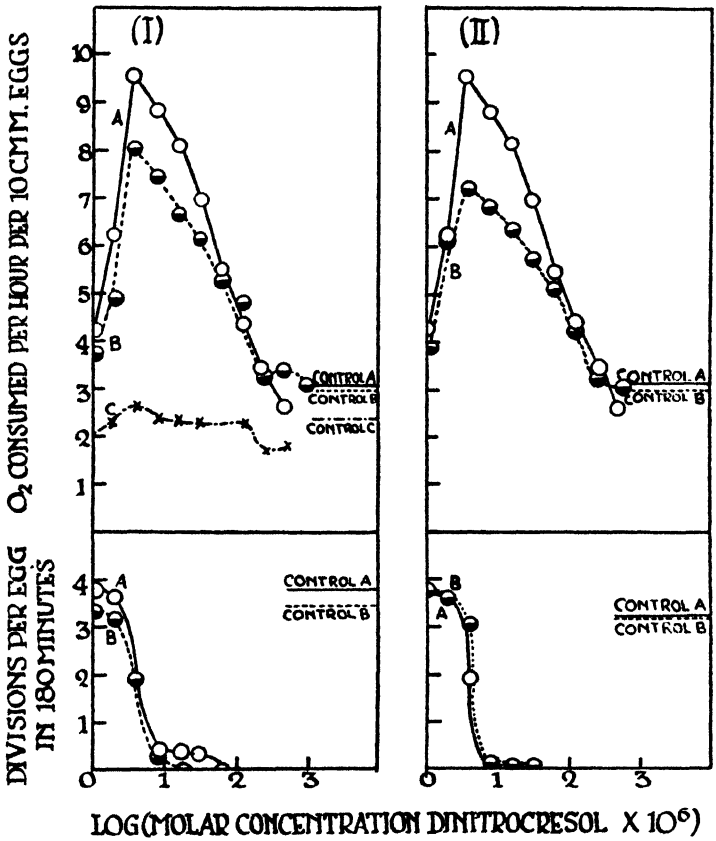

FIG. 3. Effect of various concentrations of 4,6-dinitro-*o*-cresol on oxygen consumption and cell division of *Arbacia* eggs: I*A* and II*A*, with no other reagent; I*B*, in 10^{-3}M 5-isoamyl-5-ethyl barbituric acid; I*C*, in 2×10^{-3}M 5-isoamyl-5-ethyl barbituric acid; II*B*, in 10^{-3}M malonic acid.

The reagents were added 20-30 minutes after fertilization. Temperature, 20°C.

acid had any marked effect on cell division, regardless of whether or not 4,6-dinitro-*o*-cresol was present. Greville (14) has reported that malonic acid limits respiratory stimulation by 4,6-dinitro-*o*-cresol in rat liver.

Iodoacetic Acid.—Iodoacetic acid, when used at a pH sufficiently low to allow its penetration into the eggs, produced an inhibition of respiration of sea urchin eggs as shown by Runnström (15) and confirmed in a previous

paper of this series (7). It also produced a reduction of respiratory stimulation by 4,6-dinitro-*o*-cresol which was comparable in degree to its effect on the respiration of eggs untreated with substituted phenol. Its slight inhibitory effect on cell division was additive to that of the 4,6-dinitro-*o*-cresol (Table VII). Bodine and Boell (8) have observed that iodoacetic acid limits respiratory stimulation by 2,4-dinitrophenol in orthopteran embryos.

TABLE VII

Oxygen Consumption (C.Mm. Per Hour Per 10 C.Mm. Eggs) and Cell Division (Divisions per Egg in 175 Minutes) of Fertilized Eggs of Arbacia punctulata in 8 × 10^{-6} M 4,6-Dinitro-o-Cresol with and without Various Concentrations of Iodoacetic Acid. Iodoacetic Acid Was Added at 25 Minutes after Fertilization, the 4,6-Dinitro-o-Cresol at 55 Minutes. The pH Was Approximately 6.0. Temperature, 20°C.

Concentration 4,6-dinitro-*o*-cresol	Concentration iodoacetic acid	O_2	Division
moles per l. × 10^6	*moles per l.* × 10^4		
0	0	3.0	1.88
0	2.1	2.7	2.20
0	4.2	2.4	2.05
0	8.4	2.4	1.82
0	16.8	2.0	1.05
0	33.6	1.6	0.12
8	0	5.9	0.56
8	2.1	5.0	0.35
8	4.2	5.4	0.30
8	8.4	5.1	0.31
8	16.8	3.6	0.18
8	33.6	1.2	0.08

Non-Stimulating Phenols.—2,4-dinitrothymol and 4-nitrocarvacrol differ from all other substituted phenols so far investigated in that they have been found to produce, in very small concentrations, a reversible inhibition of the cell division of fertilized *Arbacia* eggs without producing, in any concentration, a stimulation of oxygen consumption (2, 10). This appears to afford further evidence that the division inhibition by substituted phenols is associated with a depression and not a stimulation of respiratory processes. Although 2,4-dinitrothymol and 4-nitrocarvacrol produced only a moderate reduction of normal respiration, the former, at 1.6 × 10^{-6}M, produced a complete block of respiratory stimulation by 4,6-dinitro-*o*-cresol or by 2,4,5-trichlorophenol; the latter, at 4 × 10^{-6}M, likewise produced a complete block to respiratory stimulation by 4,6-dinitro-*o*-cresol (Fig. 4).

Another substituted phenol, *o*-nitrophenol, produced no inhibition of cell division and no inhibition of oxygen consumption. This substance in concentrations up to 2 × 10^{-3}M (*i.e.*, a thousand times the effective con-

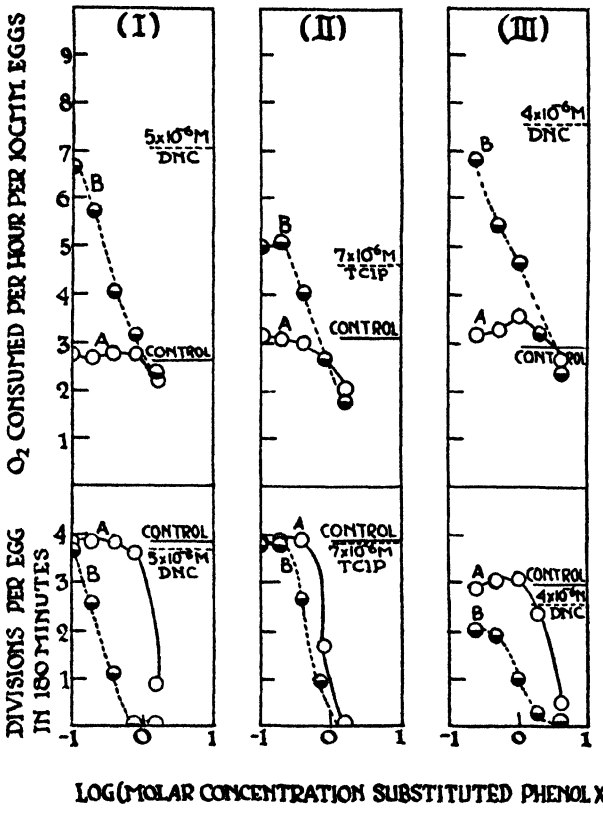

FIG. 4. Effect of various concentrations of non-stimulating phenols on oxygen consumption and cell division of fertilized *Arbacia* eggs with and without a fixed concentration of a stimulating phenol: I*A*, 2,4-dinitrothymol; I*B*, 2,4-dinitrothymol and 5 × 10^{-6}M 4,6-dinitro-*o*-cresol (DNC) together; II*A*, 2,4-dinitrothymol; II*B*, 2,4-dinitrothymol and 7 × 10^{-6}M 2,4,5-trichlorophenol (TCLP) together; III*A*, 4-nitrocarvacrol; III*B*, 4-nitrocarvacrol and 4 × 10^{-6}M 4,6-dinitro-*o*-cresol together.

All reagents were added 20–30 minutes after fertilization. Temperature, 20°C.

centration of 2,4-dinitrothymol) produced only a barely detectable reduction of respiratory stimulation by 4,6-dinitro-*o*-cresol.

The division inhibiting effects of 2,4-dinitrothymol and 4-nitrocarvacrol were additive to those of 4,6-dinitro-*o*-cresol; *o*-nitrophenol, which alone has no inhibiting effect on division, displayed in conjunction with 4,6-dinitro-*o*-cresol a very slight inhibiting effect comparable in degree with the very

small inhibiting effect which it had on 4,6-dinitro-*o*-cresol respiratory stimulation.

DISCUSSION AND SUMMARY

The effects of 4,6-dinitro-*o*-cresol and 2,4,5-trichlorophenol on the respiration and cell division of fertilized eggs of *Arbacia punctulata* have been determined in the presence of each of a number of respiratory inhibitors. The experimental results obtained appear to afford some understanding of the mechanism of action of the substituted phenols on respiration and on cell division.

1. From the fact that the stimulated respiration is completely cyanide and carbon monoxide sensitive, it may be concluded that all of the extra oxygen uptake induced in *Arbacia* eggs by 4,6-dinitro-*o*-cresol passes through the metal containing oxidase system. All of the extra oxygen uptake also passes through oxidative steps which can be poisoned by non-stimulating phenols like 2,4-dinitrothymol and 4-nitrocarvacrol, by phenylurethane, by 5-isoamyl-5-ethyl barbituric acid, by malonic acid, or by iodoacetic acid. To abolish all respiratory stimulation by suboptimum concentrations of 4,6-dinitro-*o*-cresol, each of these inhibitors must be present in a concentration which reduces the normal respiration in the absence of substituted phenols by at least 20–40 per cent.

2. The degree of reduction of the stimulated respiration by a given concentration of carbon monoxide or potassium cyanide depends on the concentration of 4,6-dinitro-*o*-cresol or 2,4,5-trichlorophenol, being most marked in suboptimum concentrations and least marked in greater than optimum concentrations of the substituted phenol. In contrast to this result, the reduction of the stimulated respiration by a given concentration of 5-isoamyl-5-ethyl barbituric acid or malonic acid is least marked in suboptimum concentrations and most marked in greater than optimum concentrations of the substituted phenol.

3. The present experiments appear to indicate that the inhibition of cell division by substituted phenols is not attributable to a direct action of these agents on mitotic processes nor to an overstimulation of any respiratory process. The inhibition of cell division appears to be associated with the inhibition, by the substituted phenols, of some component of the cyanide sensitive respiratory system. This inhibition is of such a type as to allow the overall respiration to proceed at a rate in excess of the control value, even when division is completely suppressed. The dependence of the division mechanism on a respiratory step which is relatively hypersensitive to poisoning by the substituted phenols is comparable to the depend-

ence of the Pasteur reaction in certain normal and tumor tissues on an oxidative step which is specifically poisoned by the substituted phenols (16).

The substituted phenols have no inhibiting effect *in vitro* on the principal metal containing respiratory catalysts or the principal dehydrogenases; they also do not inhibit the fermentative reactions involved in the anaerobic glycolysis of fertilized *Arbacia* eggs. It is therefore suggested that the respiratory inhibiting and division inhibiting effects of the substituted phenols may be attributable to the action of these substances on one or more of the oxidation-reduction or phosphorylating steps which are involved in the transfer of hydrogen from the dehydrogenase systems to the specifically cyanide sensitive oxidase mechanism of the eggs. The identification of the respiratory step poisoned by the substituted phenol would constitute an interesting contribution to the chemistry of cell division and experiments to this end are now in progress.

The authors are indebted to Dr. John F. Taylor and to Dr. John O. Hutchens for their cooperation in the performance of certain of the experiments here reported.

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SOME CHEMICAL ASPECTS OF THE POTASSIUM EFFECT

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The ability to distinguish electrically between Na^+ and K^+ (potassium effect) appears to exist in *Nitella* only so long as it contains a group of organic substances which has been called for convenience R_P.[1] As this is removed from the cell by treatment with distilled water the potassium effect falls off.

Earlier experiments indicate that the potassium effect depends on the mobility ratio[2] $u_K \div u_{Na}$ and on the partition coefficient[3] ratio $S_K \div S_{Na}$ (S_K is the ratio K^+ plus $(KX)^+$, etc., in the non-aqueous protoplasmic surface layer $\div$ K^+ in the external solution).

If both ratios depended on a single substance an increase in the potassium effect would mean an increase in both ratios. But we find that these ratios can change in opposite directions. Hence we infer that they depend on different substances contained in R_P.

It is convenient to assume that two substances, R_{MK} and R_{SK}, increase the mobility and partition coefficient respectively of K^+ and that two others, R_{MNa} and R_{SNa}, do the same for Na^+. It should be understood, however, that this assumption is made chiefly for purposes of discussion. As will appear later, there is an obvious distinction between the substances affecting K^+ and those affecting Na^+, but to what extent R_{MK} differs from R_{SK} or R_{MNa} differs from R_{SNa} is less certain.

The fact that the ratio $u_K \div u_{Na}$ can change in the opposite direction from $S_K \div S_{Na}$ is shown by recent measurements on cells (Lot B[4])

[1] Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 107.

[2] u_{Na} and u_K are really $u_{Na} \div V_{Cl}$ and $u_K \div V_{Cl}$: here V_{Cl} is taken as unity.

[3] Regarding $(KX)^+$, etc., see Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 34; Kraus, C. A., *Tr. Electrochem. Soc.*, 1934, **66**, 179; Fuoss, R. M., *Chem. Rev.*, 1936, **17**, 27.

[4] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) for 22 days. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312) unless otherwise stated.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). Temperature about 26°C.

collected during the summer and early fall,[5] which yield the following values.

For the concentration effect,[6] 0.01 M followed[7] by 0.001 M KCl, we find 26 ± 2.3 mv. (10 observations). From this we get[8] $u_K = 2.62$.

For the concentration effect of NaCl, *i.e.* 0.001 M followed by 0.01 M and *vice versa*, we have 20 ± 0.8 mv. (12 observations). From this we get $u_{Na} = 2.05$.

On substituting 0.01 M NaCl for 0.01 M KCl the change[9] of P.D. is 55 ± 2.4 mv. (12 observations). To account for this we assume that $S_K \div S_{Na} = 100$.

Cells collected during the winter[8] give the following values: $u_K \div u_{Na} = 11.9 \div 7.93 = 1.5$ and $S_K \div S_{Na} = 60$.

In the summer cells we have $u_K \div u_{Na} = 2.62 \div 2.05 = 1.28$ and $S_K \div S_{Na} = 100$. Hence in the summer cells the mobility ratio has decreased from 1.5 to 1.28 and the partition coefficient ratio has increased from 60 to 100 as compared with the winter cells.

In experiments on winter cells we find that u_K varies independently of u_{Na} since the ratio $u_K \div u_{Na}$ varies. Hence we infer that R_{MK} varies independently of R_{MNa}. Also R_{SK} may vary independently of R_{SNa} since S_{Na} may rise while S_K remains constant. This is shown by the following measurements.

When winter cells of Lot A[10] are treated with distilled water, u_K decreases[11] from 85 to about 2 and u_{Na} remains unchanged at about 2. Hence we may infer that R_{MK} is leached out while R_{MNa} is little affected. We find that $S_K \div S_{Na}$ may fall to less than unity so that 0.01 M KCl becomes

[5] During this season, when growth is relatively rapid, the mobility of K^+ is frequently low, suggesting a deficiency of R_{MK}, such as is brought about in winter by leaching R_{MK} out of the cells by placing them in distilled water (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 987).

[6] The dilute solution is positive in the external circuit.

[7] When 0.001 M is followed by 0.01 M KCl, action currents may occur which make the change in P.D. unduly large (*cf.* Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541).

[8] For the method of calculation see Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. It should be noted that as here used, $u_K = u_K \div V_{Cl}$ and $u_{Na} = u_{Na} \div V_{Cl}$; V_{Cl} is taken as unity in both cases.

[9] When 0.01 M NaCl is followed by 0.01 M KCl action currents may occur which make the change in P.D. unduly large.

[10] *Cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. See footnote 4.

[11] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; 1934–35, **18**, 993.

positive to 0.01 M NaCl. In winter cells of Lot B, treated with distilled water, $u_K \div u_{Na}$ may fall[12] from $11.9 \div 7.93 = 1.5$ to $2.22 \div 2.22 = 1$ and $S_K \div S_{Na}$ from 60 to 0.12. Hence we may infer that R_{MK} is leached out of the cell more than R_{MNa} and that leaching must affect R_{SK} much more than R_{SNa} to lower the ratio $S_K \div S_{Na}$ so greatly.

In winter cells of Lot B[10] guaiacol[13] raises R_{SNa} much more than R_{MNa} and since it raises the mobility and partition coefficient of Na^+ but not of K^+ we may say that R_{MNa} and R_{SNa} increase but not R_{MK} and R_{SK}.[14]

It may be noted, in passing, that the treatment with distilled water, which presumably brings about a loss of substances, never produces an effect which has to be interpreted as a rise in mobility or in partition coefficient. Hence we need not assume the presence of substances which depress the values.

These results are not altogether surprising in view of experiments with models[15] which show great differences in mobilities and partition coefficients in various non-aqueous substances. The differences in S_K doubtless depend largely on the fact that the potassium entering the non-aqueous protoplasmic surface layer may enter into new combinations and thus change the partition coefficient which, as used here, is the ratio of K^+ in the external solution[16] to any form of potassium which could affect the potential, *e.g.* K^+, $(KX)^+$, etc.

Certain organic compounds of potassium are very soluble in non-aqueous media[17] and if these are present in the non-aqueous protoplasmic surface they may give a high value to S_K and this[18] may be greater than for S_{Na}. It is evident that u_K and u_{Na} might be changed by substances which affect viscosity, solvation or the formation of $(KX)^+$, $(NaX)^+$, etc.

There is not much prospect of obtaining sufficient R_P for chemical analysis since it is presumably present only in traces. But certain experi-

[12] Osterhout, W. J. V., and Hill, S. E., *Proc. Nat. Acad. Sc.*, 1938, **24**, 427.

[13] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 171.

[14] This is also the case with u_K and u_{Na} in *Halicystis* (Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 53) but in *Valonia*, u_{Na} increases and u_K decreases (Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13).

[15] Unpublished results.

[16] Strictly speaking the partition coefficient of a single ion has no physical meaning but it is permissible to use it in description as in the present case.

[17] Hundeshagen, F., *J. prakt. Chem.*, 1883, **28** N.S., 219.

[18] In guaiacol S_K is greater than S_{Na} (*cf.* Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 469).

ments by S. E. Hill indicate that R_P or one of its essential constituents is soluble in petroleum ether.[19]

Other experiments suggest that R_P or one of its essential constituents is a potassium compound. Certain cells which have lost the potassium effect by leaching in distilled water soon regain the potassium effect when placed in contact with KCl.[1] The delay might be due to the time required[20] for the combination of potassium with an organic compound in the protoplasm to produce the potassium effect. If this organic compound has been leached out of the cell by the distilled water the combination cannot occur and the application of potassium cannot restore the potassium effect: this seems to happen in most cases. Regarding this organic compound not much can be said but it appears to be present in blood which can restore the potassium effect in a few seconds.[21]

It may be added that *Nitella* contains another substance (or group of substances) called for convenience R_A which enables the cell to give action currents when stimulated electrically.[1]

SUMMARY

The ability of *Nitella* to distinguish electrically between Na^+ and K^+ (potassium effect) appears to depend on several organic substances (or groups of substances). Of these R_{MK} and R_{SK} determine the mobility and partition coefficient (S) respectively of K^+ while R_{MNa} and R_{SNa} do the same for Na^+.

These substances can vary independently and this variation is susceptible to experimental control.

[19] Experiments in this laboratory by S. E. Hill show that the water in which *Nitella* has been standing contains substances which are taken up by petroleum ether when shaken with the water. The petroleum ether is evaporated and the residue dissolved in water. When this is applied to a cell which has lost its potassium effect as the result of leaching in distilled water (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 105) the potassium effect is promptly restored.

[20] It seems possible that this reaction is catalyzed by ammonia or its compounds, or they may enter into the formation of the organic substance, *cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 987.

[21] Osterhout, W. J. V., *J. Gen. Physiol.*, 1935–36, **19**, 423.

STUDIES ON PITUITARY LACTOGENIC HORMONE*

I. Electrophoretic Behavior

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INTRODUCTION

The importance of the technique of electrophoresis to protein chemistry has rapidly grown since Tiselius (1) described the method in 1937. Proteins, such as ovamucoid (2) and prolan (3), which are not crystalline substances, have been shown to be highly purified and homogeneous in the Tiselius apparatus. On the other hand, the fact that many samples of crystalline pepsin are not homogeneous has been confirmed by electrophoresis measurements (4). In our studies, the Tiselius technique has been used to determine the homogeneity of preparations of pituitary lactogenic[1] hormone, and to determine the isoelectric point of the hormone, which has not heretofore been obtained with sufficient accuracy by the electrophoretic method.

EXPERIMENTAL

Lactogenic Hormone Preparations

The lactogenic hormone L 269 was prepared in essentially the same manner as previously published (5, 6). The final product was precipitated out of an aqueous solution made slightly alkaline with NaOH, by adding HCl until the pH approximated 5.5. An acetone-dried powder was made from this precipitate. Preceding an electrophoresis experiment, the powder was dissolved in water made slightly acid or alkaline, and dialyzed against the desired buffer mixture. By direct comparison with a standard prolactin powder LP 1 (10 units per mg.) supplied by Drs. Riddle and Bates to the League of Nations and kindly sent to us by Dr. A. S. Parkes, L 269 was found to have approximately 20 Riddle-Bates units per mg. L 269 also caused lactation in normal, virgin, post-estrus guinea pigs in a total dose of 4 mg. or less.

* Aided by grants from the Board of Research of the University of California, from The Rockefeller Foundation, from Parke, Davis and Company, and from the National Research Council Committee on Research in Endocrinology. Assistance was rendered by the Works Progress Administration, Project No. OP 665-08-3-30, Unit A-5.

[1] The term "lactogenic" is employed for convenience for the crop gland stimulating hormone.

Apparatus

All parts of the Hellige apparatus employed by us are the same as that described by Tiselius (1). A diagram indicating the arrangement of the apparatus is shown in Fig. 1. The principle of the schlieren method for observations of the boundaries and the construction of the electrophoresis cell have been described recently by MacInnes (7). The image of the slit *S*, illuminated by the lamp *L* condenser *C*, is projected into the schlieren diaphragm *D* by the Dallmeyer objective *P* which is placed as near to the electrophoresis cell *E* as possible. The camera lens *O* is focused on the cell and forms a 7/10 size image on a frosted glass plate or photographic plate (Eastman Kodak, orthochromatic, 4.5 × 6 cm.). The electrophoresis cell and electrode vessels are in a thermostat which is kept at 3.0 ± 0.2°C. by Linde cooling aggregate type "multifrigor" with automatic cooling device. The water in the thermostat is circulated by means of a ¼ h.p. centrifugal pump instead of a mechanical stirrer which generally produces some vibrations in the system. An electrical unit, consisting of a center-tapped 3000 volts transformer with two rectifying tubes, is used for the current supply. The voltage thus obtained has small fluctuations and the current is practically constant throughout the experiment.

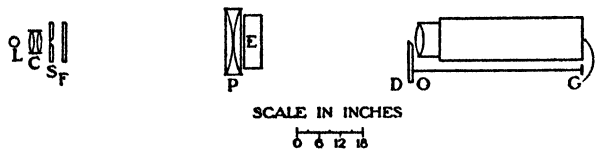

FIG. 1. Arrangement of the electrophoresis apparatus: *L*, H4 type mercury vapor lamp operated with a special transformer; *C*, projection condenser; *S*, horizontal slit; *F*, Wratten filter No. 77; *P*, Dallmeyer objective of *f* about 60 cm. with 15 cm. diameter; *E*, electrophoresis cell, and *D*, schlieren diaphragm; *O*, camera objective (1:7.7 *f* = 45 cm.); *G*, frosted glass plate with millimeter scale or photographic plate.

Measurements

For homogeneity studies, 0.5 to 1.0 per cent lactogenic hormone solutions were used. Before putting the protein solutions into the electrophoresis cell, the solutions were dialyzed against the buffers for at least 24 hours until there was no difference in the pH and conductance between the dialysate and the buffer. This is particularly important because boundary anomalies (8) arise from the differences in specific conductance and hydrogen ion concentration between the protein and buffer solutions. The homogeneity of the protein in the course of the electrophoresis was observed carefully by the image in the frosted glass plate by adjusting the movable schlieren diaphragm, and recorded photographically at intervals.

The usual Wheatstone bridge type of circuit and a Washburn conductivity cell were employed in measuring the specific conductance of the solution at the same temperature as that at which the electrophoretic experiments were performed. A Leeds and Northrup type S.P. 929 bridge was used, the resistance of the solution being balanced against that of a L. and N. 99,990-ohm, 4-dial resistance box by means of an L. and N. type (c) A.C. galvanometer, the deflections being observed through a wide angle lens. 110-volt, 60-cycle A.C. was used to activate the coils of the galvanometer and 6-volt, 60-cycle

A.C. was used in the bridge circuit. The hydrogen ion concentrations of the protein and buffer solutions were determined with the glass electrode.

For mobility determinations, two well fitted rubber stoppers were used to reduce the free solution area in the electrode tubes so that no hydrostatic displacement occurred in the U-tube. The movement of the boundaries during electrophoresis was followed by recording the position of the sharp line in the frosted glass plate at intervals. The average displacement of the boundary per second was multiplied with the reduction factor (1.43) in order to give the apparent speed of the boundary.

The buffer solutions of ionic strength 0.055 used in all experiments were prepared by sodium acetate acetic acid and potassium dihydrogen phosphate sodium monohydrogen phosphate mixtures.

Mobility Calculation

The mobility (u) is generally calculated by a simple equation: $u = S/F$, where S is the apparent speed of the boundary as defined above and F, the potential gradient (9), which is determined by the current[2] i ampere, cross-section of the electrophoresis cell q sq. cm., and the specific conductance of the solution λ: $F = i/q\lambda$. The cross-section of the electrophoresis cell was determined from the calibration with mercury and found to be 0.810 sq. cm. The mobility thus obtained has the dimension centimeter per second per volt per centimeter of the order of magnitude 10^{-5}.

RESULTS AND DISCUSSION

The protein concentrations[3] used for homogeneity study were 0.5 and 1.0 per cent solutions. The schlieren photographs, taken at 15 minute intervals of a typical experiment with 0.5 per cent solution, are shown in Fig. 2. They showed one and only one sharp boundary throughout the electrophoresis experiments. Since Tiselius (10) has shown that 0.02 per cent protein solution can be detected by electrophoresis, the single boundary shown by our lactogenic preparation indicates that in all probability no contaminant exists in greater amount than 0.02 per cent. It might also be argued that possible contaminants had nearly the same mobility as the main component so that resolution into two bands is not permitted.

These lactogenic hormone solutions were then diluted to 0.2 to 0.3 per cent for mobility studies. The potential gradients were kept practically constant in all experiments (*ca.* 9 volts/cm.). The results were recorded

[2] The current is measured by a millimeter which is calibrated against Leeds and Northrup 7560 portable potentiometer standardizing set.

[3] The protein concentration was determined after dialysis. It was obtained by multiplying the nitrogen content, which was determined by micro-Kjeldahl analysis, with the factor 6.33 assuming the lactogenic hormone contains 15.8 per cent nitrogen.

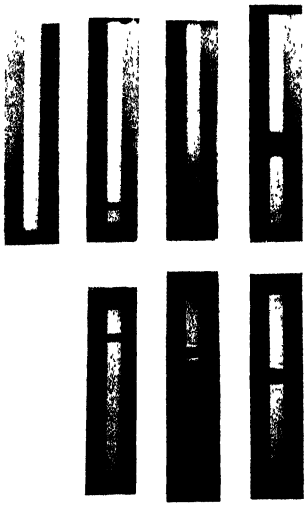

FIG. 2 A typical example of schlieren photographs of the electrophoresis of pituitary lactogenic hormone. Exposures were made at 15 minute intervals.

in Table I. The plus and minus signs refer to the charge on the protein. It will be seen in Fig. 3 that the isoelectric point of pituitary lactogenic hormone is 5.70. This value is in accordance with our observations that the maximum precipitation of the hormone occurs at about pH 5.5. It should be noted that the $\frac{du}{d\text{pH}}$ value of the curve is 4.5×10^{-5}, which is in agreement with the prediction of Tiselius (11).

In their recent report Shipley, Stern, and White (12) have determined the mobility of their crystalline lactogenic hormone at 4 hydrogen ion concentrations. It is of interest to note that only one of their measurements at pH 6.52 ($u = -3.64 \times 10^{-5}$ cm.^{2}/sec./volt) checks closely with our value. The material used for this measurement is said by them to be denatured by storage at low temperature.

TABLE I

Electrophoretic Mobilities of Pituitary Lactogenic Hormone at 3°C. in Acetate and Phosphate Buffers of Varying pH and Constant Ionic Strength (0.055)

Buffer	pH	Mobility in cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^5$
Acetate	3.75	+8.70
"	4.00	+7.07
"	4.30	+6.31
"	4.75	+5.00
Phosphate	6.50	−3.39
"	6.80	−5.32
"	7.00	−5.75
"	7.20	−6.50
"	7.85	−10.00

Slope of curve at isoelectric point, $\frac{du}{d\text{pH}} = 4.5 \times 10^{-5}$, isoelectric point = 5.70.

The difference in mobility of their undenatured crystalline and our non-crystalline hormone indicates that the two substances are not identical and might suggest that our lactogenic hormone is a denatured product, but even

granting that our material has been denatured in the process of purification, it is remarkable that it does not show biological deterioration for it possesses the highest potency yet attained by us and has at least twice the potency found in a crystalline preparation kindly sent us in 1938 by Doctor White.

To test whether or not the lactogenic hormone can be denatured by *storage*, two experiments were carried out as nearly as possible in the manner described by Shipley *et al.*[4] No difference whatsoever was found in the mobility and homogeneity of the fresh and stored hormone solutions. It should be mentioned that during the dialysis of the hormone solution in pH 6.5 phosphate buffer, no turbidity or precipitation was observed in the dialysate. This is very important because a possible contaminant in any lactogenic preparations is the adrenotropic hormone which shows maximum precipitation at about pH 6.5. Furthermore, the solutions which were dialyzed between pH 4 and 5 became turbid, yet this did not interfere with the optical observations in electrophoresis experiments.

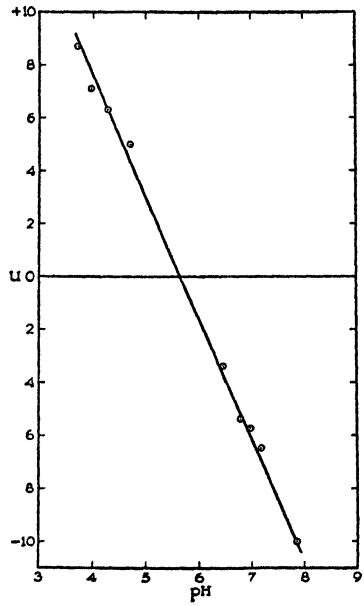

FIG. 3. Mobility of pituitary lactogenic hormone at different pH's (temperature 3°C.).

The crop gland stimulating action of the hormone, which was used over a 10 day period in four electrophoresis experiments, was recovered by acetone precipitation and tested again biologically. No change from its original potency was detected.

SUMMARY

The Tiselius technique of electrophoresis has been used to study the homogeneity and mobility of pituitary lactogenic hormone. The lactogenic preparation employed by us shows only one sharp band in schlieren photographs, indicating a high degree of homogeneity. From the deter-

[4] It is very difficult to determine from the paper of Shipley, Stern, and White what concentration of the protein solution is mixed with 1.5 cc. 0.5 N KOH solution. We added 1.5 cc. 0.5 N KOH into 10 cc. 0.3 per cent protein solution which had been frozen at −18°C. for 2 days. We let the solution stand at room temperature for a few minutes in one experiment and 1 hour in another experiment.

mination of the mobility of the hormone at different hydrogen ion concentrations in acetate and phosphate buffer of ionic strength 0.055, the isoelectric point was found to be 5.70 and the $\frac{du}{d\text{pH}}$ value 4.5×10^{-5}.

The difference in the mobility reported for White's crystalline preparation and that found with our high potency lactogenic preparation is discussed.

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ELECTROPHORESIS OF PEPSIN

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(Received for publication, December 4, 1939)

The first samples of crystalline pepsin were found to be a protein contaminated with 5–10 per cent non-protein material (1). On standing in solution the protein is hydrolyzed, the activity decreases, and this non-protein material increases more or less rapidly depending on the conditions (2). Solubility experiments (1) showed that the protein component of this preparation was at least as pure as the common proteins previously crystallized but showed some indications of the presence of several closely related proteins.[1] This is the case with all proteins so far studied with the possible exception of chymotrypsinogen (14). These experiments were all made with one particular lot of commercial pepsin. Subsequent work in this and other laboratories has shown that crystalline pepsin prepared from different sources may differ in activity, stability, isoelectric point, and solubility. Thus, Northrop (3) found that pepsin crystals prepared from certain samples of commercial pepsin contained nearly 50 per cent inert protein. All samples of once crystallized pepsin contain more or less of another enzyme which acts on gelatin (4). Herriott (5) showed that pepsin prepared from pepsinogen and from various commercial preparations differed 10–30 per cent in activity. Holter (6) obtained some indication of enzymes of different activities by means of adsorption experiments. Dyckerhoff and Tewes (17) also found slight variations in activity. Ågren and Hammarsten (7) obtained an increase in activity as calculated on a total nitrogen basis by electrophoresis and their results have been confirmed by Tiselius, Henschen, and Svensson (9). Steinhardt (8) found a variation in solubility of pepsin at pH 2.7 and suggested that the effect was due to the non-protein material present. Solubility studies (10) show that these differences are probably due to the fact that there are several active proteins

* Fellow of the Belgian American Foundation.

[1] It may be recalled that these determinations were made on samples which had been treated by a series of extractions in the solvents used and not on crystals direct from the mother liquor.

and that the differences in the various commercial preparations are due to differences in the proportion of the various components present.

It has been possible to prepare pure "A" pepsin of constant solubility from pepsinogen and also from certain especially active commercial prepara-

TABLE I

Electrophoresis of Various Pepsin Solutions

Exp. No.	Pepsin preparation	Cell used	pH	Distance of migration	Moving boundary	Stationary boundary	Per cent N.P.N.		$[P.U.]^{Hb}_{Total\ N}$		$[P.U.]^{Hb}_{Protein\ N}$	
							Orig.	Anode	Orig.	Anode	Orig.	Anode
				cm.								
1*	Glycerine standard dialyzed	Tiselius	4.6	2	Sharp	—	3	3	0.29	0.29	0.29	0.31
2*	Same as No. 1	"	4.43	8	"	—	4	3.3	0.30	0.30	0.30	0.305
3	Same as No. 1	U tube	4.6	8	"	Faint	5	5.0	0.28	0.28	0.30	0.30
4	Glycerine standard dialyzed and partly inactivated	"	4.6	2	Diffuse	"	18	13.0	0.25	0.29	0.29	0.31
5	Same as No. 4	"	4.6	4	"	"	30	20.0	0.19	0.26	0.28	0.33
6	Same as No. 4	"	4.6	10	"	"	16	13.0	0.25	0.27	0.31	0.33
7	Crystallized from Cudahy, dialyzed	Theorel	4.1		—	"	12	1.5	0.26	0.29	0.29	0.29
8	Same as No. 7	U tube	4.6	6	Sharp	"	10	5.0	0.26	0.30	0.29	0.31
9	Same as No. 7	"	4.6	9	"	"	10	5.0	0.26	0.28	0.29	0.28
10	Same as No. 7	"	4.6	8	"	"	15	3.0	0.25	0.30	0.29	0.31
11	Same as No. 7	M cell	4.6	3	"	"	15	4.0	0.26	0.29	0.29	0.30
12	Crystalline Parke, Davis	U tube	4.6	9	"	—	5	1.5	0.23	0.24	0.24	0.24
13†	Pure "A" pepsin	Tiselius	4.6	2	"	—	2	2.0	0.33	0.33	0.33	0.33
14	Mixture: 0.44 activity pepsin + 0.28 activity pepsin	U tube	5.2	5	"	—	10	10.0	0.31	0.32	0.34	0.36

* The electrophoresis in these experiments was carried out by Dr. Alexandre Rothen, Laboratory of Physical Chemistry at the Rockefeller Institute.

† The electrophoresis was carried out by Dr. Lewis G. Longsworth, Laboratory of Physical Chemistry at the Rockefeller Institute.

tions (Herriott, Desreux, and Northrop, unpublished experiments). Another component of low activity 0.1 $[P.\ U.]^{Hb}_{mg.\ P.N.}$ which has an isoelectric point at pH 4.0 has also been isolated (Desreux and Herriott). No general method has been found so far, however, for separating the mixtures into

their various components. Ågren and Hammarsten (7) and Tiselius, Henschen, and Svensson (9) have found that electrophoresis of pepsin solutions yields a substance of higher activity than the original solutions as calculated on a total nitrogen basis. According to Tiselius *et al.* this is a protein, shows a sharp boundary, and migrates as a homogeneous substance. It seemed possible, therefore, that the electrophoresis method might furnish a means of separating the various protein components of a pepsin solution. A number of electrophoresis experiments were therefore carried out with various pepsin preparations. The results of these experiments are summarized in Table I.

The results show that there is no separation of the protein components from each other but that they migrate at the same rate and show a homogeneous boundary. This accounts for the fact, noted by Tiselius, that the specific activity of the migrating component is different in different preparations. More or less of the non-protein nitrogen is left behind at pH 4.4 and the migrating protein therefore has a higher activity on a total nitrogen basis than the original solution, as Tiselius and his collaborators and Ågren and Hammarsten stated. There is, however, no increase in activity on a protein nitrogen basis.

The "glycerine standard" pepsin of Table I is the same solution as that used by Tiselius. It is a twice crystallized preparation from Cudahy pepsin. The solubility diagram of this preparation is shown in Fig. 1. The diagram shows that more than one protein is present. The migrating boundary, however, is homogeneous (Fig. 5) as Tiselius states but this migrating material still contains more than one protein component.

The solubility diagram of another sample of crystalline pepsin prepared from Cudahy pepsin is shown in Fig. 2 and that of pure "A" pepsin[2] in Fig. 3. The electrophoresis of a sample of pure "A" pepsin is reported in experiment 13 and Fig. 4, and that of the Cudahy sample in experiments 7–11 of Table I. The solubility diagrams show that the crystalline Cudahy pepsin contains several proteins while the "A" pepsin contains only one. The electrophoresis shows only one boundary in both samples.

The electrophoresis of a mixture of highly active pepsin (specific activity 0.44 P.U.) and a fraction of low activity (0.28) is shown in experiment 14, Table I. There is again no separation. The activity of the migrating mixture is the same as that of the original mixture.

[2] Samples of "A" pepsin recently isolated give a total solubility in this solvent of 0.85 mg. N/ml. instead of 0.65 mg. This difference may be due to slight differences in salt concentrations or to the fact that the sample reported still contained small amounts of a more insoluble protein present as a solid solution.

Electrophoresis at pH 3.5.—In the preceding experiments at pH 4.1–5.2 the protein migrates to the anode and the non-protein nitrogen remains

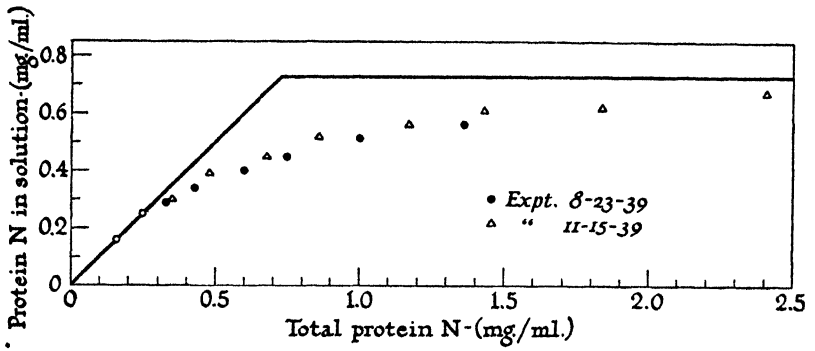

FIG. 1. Solubility diagram of "standard glycerine" pepsin in half saturated magnesium sulfate 0.05 M pH 4.6 acetate at 22°C.

FIG. 3

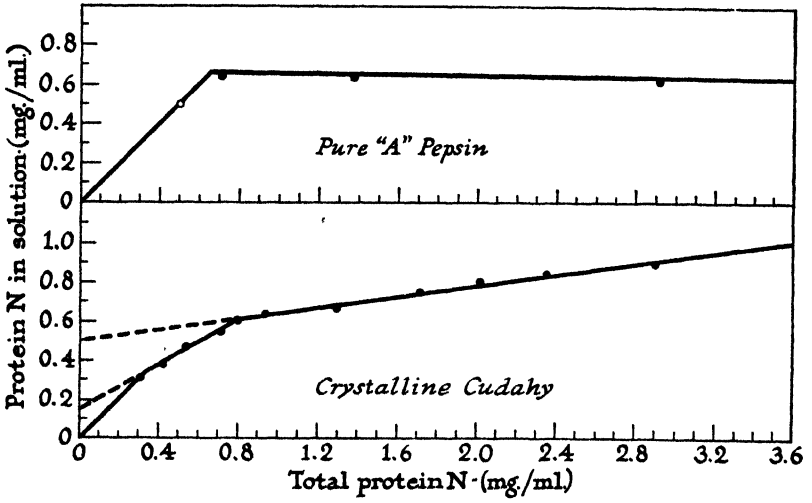

FIG. 2

FIG. 2. Solubility diagram of crystalline pepsin prepared from Cudahy pepsin in half saturated magnesium sulfate 0.05 M pH 4.6 acetate at 22°C.

FIG. 3. Solubility diagram of pure "A" pepsin prepared from specially active preparation of Cudahy pepsin. Conditions the same as in Figs. 1 and 2.

stationary. At pH 3.4, however, some of the non-protein nitrogen migrates to the cathode and the protein to the anode. This confirms the results of Ågren and Hammarsten, Table II. There is, however, no change in the

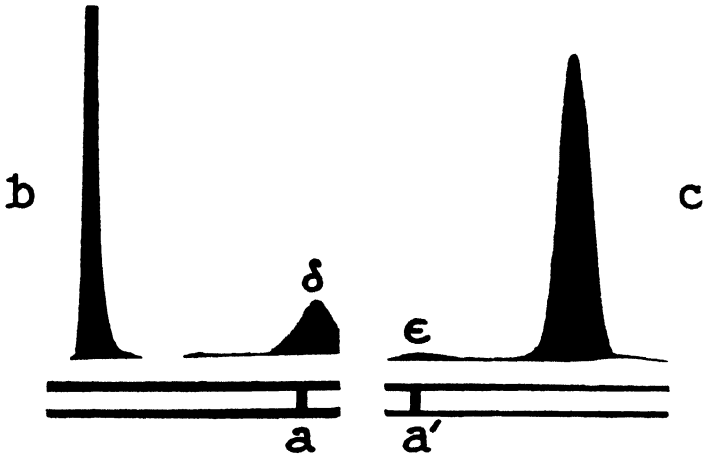

FIG. 4. Electrophoretic patterns at 0°C. of pure "A" pepsin in an 0.014 N sodium acetate buffer at pH 4.46. *aa′*, positions in the cell to which the boundaries were shifted before the potential was applied. *b*, *c*, patterns of the rising and descending boundaries, respectively, after 4000 sec. at 6.9 volts/cm. The rising boundary was still too sharp to be recorded completely. The δ and ε boundaries are concentration gradients remaining near the initial boundary positions and *do not* represent electrically inert material. The mobility computed from the displacement of the descending boundary is $u = -7.5_3 \times 10^{-5}$ cm.^{2}/volt/cm.

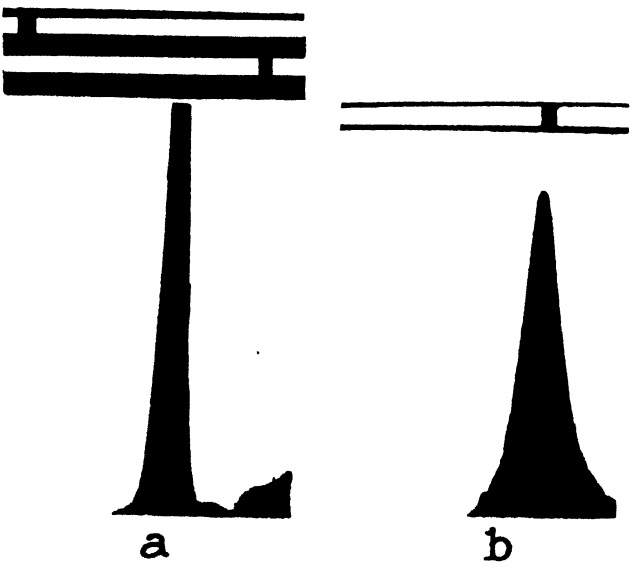

FIG. 5. Longsworth pattern of dialyzed "standard glycerine" twice crystallized Cudahy 12–7–33 pepsin at pH 4.43, and 3.3 volt/cm. (*a*) Rising anode boundary after 6900 seconds; 1.28 cm. migration; mobility = -5.6×10^{-5} cm.^{2}/volt/sec. (*b*) Rising anode boundary after 57,300 seconds; 9.0 cm. migration; mobility = -4.7×10^{-5} cm.^{2}/volt/sec.

activity of the protein and the change in total activity observed is due merely to the separation of protein and non-protein compounds.

Isoelectric Point of Pepsin.—Ringer (11) found that most samples of pepsin, as obtained from gastric juice, migrated always to the anode, even in strongly acid solution. The addition of protein split products, however, caused the enzyme to migrate to the cathode on the acid side of about pH 3.0. Ringer concluded that the enzyme was an acid. This result was confirmed by Northrop (12) from a study of the distribution of pepsin between egg albumin particles and the surrounding solution. The distribution was found to be the same as that of the chloride ion indicating that pepsin was a negatively charged monovalent acid at least as far acid as pH 1.0.

Tiselius, Henschen, and Svensson (9) have recently obtained the same result by electrophoresis measurements. Cataphoresis measurements on

TABLE II

Exp. No.	Pepsin preparation	Cell used	pH	Distance of migration	Boundaries		Per cent N.P.N.			$[P.U.]^{Hb}_{Total\ N}$			$[P.U.]^{Hb}_{Protein\ N}$		
					Anode	Cathode	Orig.	Anode	Cathode	Orig.	Anode	Cathode	Orig.	Anode	Cathode
				cm.											
15	Crystalline Cudahy in N/10 pH 3.4 citrate	M	3.4	3	Sharp	Diffuse	10	6	70	0.27	0.30	0.10	0.30	0.31	0.30

collodion particles coated with crystalline pepsin, however, showed an isoelectric point at about pH 2.7 and a minimum solubility was observed near this point (1). This result has now been found to be due to the effect of decomposition products on the measurement as originally described by Ringer. Thus, solutions of pure "A" pepsin, when freed from non-protein nitrogen are negatively charged even in N/10 hydrochloric acid. After a few hours at 30°C., however, during which time about 5 per cent non-protein nitrogen appeared in the solution, the particles became positively charged at pH 1.5.

The fact that pepsin contains at least two primary amino groups but does not become positively charged even in 0.1 M acid indicates the presence of a very strong acid group. An indication of such a group is also found in the titration curves but cannot be determined definitely in this way. Pepsin contains 1 atom of phosphorus (1) and this acid group may be that of phosphoric acid.

EXPERIMENTAL

Pepsin Activity Measurements.—The peptic activity was determined by the hemoglobin method as recently described by Anson (15). This method is accurate to ±2 per cent when different samples are run at the same time and with the same hemoglobin solution. Determinations made at different times but with the same hemoglobin solution may vary ± 5 per cent. When the determination is made with different hemoglobin solutions and at different times the activity obtained may vary ± 20 per cent. For this reason it is necessary to run a "standard pepsin solution" with each unknown. The activity of the standard pepsin is taken as the average value obtained for that solution in a long series of determinations. The activity obtained with an unknown solution is corrected in proportion to the correction found necessary with the standard pepsin solution. That is, the activity of the unknown solution equals $\frac{SX}{A}$ when A is the observed activity of the standard solution in this particular run, S is the average value of the standard, and X is the observed activity of the unknown solution in the same series of determinations.

The glycerine standard solution used in these experiments gave a value of 0.29 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. P.N.}}$ or 0.26 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. total N}}$ over a series of determinations. For the same solution after dialysis Tiselius found 0.21 Hb. U. per mg. total nitrogen. In view of the increase in activity found by Tiselius it appears probable that the solution analyzed by him contained 20–30 per cent non-protein nitrogen and hence gave a low initial activity. Unfortunately neither Tiselius and his coworkers nor Ågren and Hammarsten analyzed for protein nitrogen. Since Tiselius obtained a value of 0.34 $[\text{P.U.}]^{\text{Hb}}_{\text{mg. total N}}$ after electrophoresis it appears that his figure of 0.34 corresponds to our figure of 0.29.

The non-protein nitrogen exists in at least two forms since part may be removed by dialysis and part cannot be removed in this way. The proportion of dialyzable non-protein nitrogen varies with different preparations and no general statement can be made concerning its occurrence.

Nitrogen Determination.—Nitrogen was determined by the micro-Kjeldahl method.

Non-protein nitrogen is defined as nitrogen that is not precipitated by boiling 5 per cent trichloracetic acid. In making the determinations it is important that a relatively small sample (1.0 – 2.0 cc.) of pepsin solution be run into 8-10 ml. of boiling trichloracetic acid. If the trichloracetic acid is not boiling or if the pepsin solution is added so rapidly as to cool the acid large amounts of non-protein nitrogen may be formed by the action of native pepsin on the denatured protein already present. Protein nitrogen is taken as the difference between total nitrogen and non-protein nitrogen. The specific activity per milligram total nitrogen is of much less significance in the case of pepsin than is the activity per milligram protein nitrogen. For example, in the crystallization of pepsin from commercial pepsin the activity per milligram total nitrogen increases about 500 per cent. Upon standing in solution the activity may decrease again many hundred per cent in a few hours or days, depending upon conditions (2). The activity per milligram protein nitrogen, however, remains practically constant through all these changes.

Electrophoresis Measurements.—Experiments 1 and 2 were performed by Dr. Alexandre Rothen and experiment 13 by Dr. Lewis G. Longsworth. The Tiselius apparatus was used in these experiments.

Experiment 7 was made in a Theorel (16) apparatus constructed of lucite.

The other experiments were made in an open U tube except experiments 11 and 15 which were done in an M shaped cell of lucite. This type of cell has proved very simple to make and has been found to give very good results. It may readily be constructed from strips of glass or of thin lucite, as shown in Fig. 6. Both large and small cells have been used. They are filled and samples are taken by means of capillary pipettes. If one arm is to be sampled without disturbing the solution the bottom of the V is filled with mercury from a capillary pipette before sampling, thus sealing off the other arm. The boundaries are observed by the method described by MacInnes and Longsworth (13).

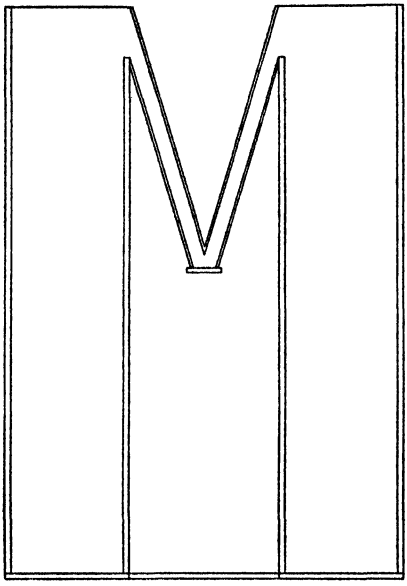

FIG. 6. M shaped electrophoresis cell of lucite.

Solubility Determination.—The solubility determinations were made by precipitation of the amorphous protein as described by Northrop (1) and the curves analyzed as described by Kunitz and Northrop (14). The protein content of the solutions and suspensions was determined by turbidity measurements (18) after precipitation of the protein by boiling 2 per cent trichloracetic acid plus 10 per cent saturated magnesium sulfate solution.

SUMMARY

1. A number of pepsin solutions containing several protein components have been studied by the electrophoresis method. All samples show a homogeneous boundary moving to the anode at pH 4.4.

2. The activity of this material may be higher than that of the original solution on the basis of total nitrogen but is the same as that of the original solution on the basis of protein nitrogen.

3. There is no separation of the various protein components under these conditions.

4. The apparent isoelectric point at pH 2.7, previously obtained by the collodion particle method is due to the presence of decomposition products. Pure crystalline pepsin, free from decomposition products, is always negatively charged.

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TIME-TEMPERATURE RELATIONS IN THE INCUBATION OF THE WHITEFISH, COREGONUS CLUPEAFORMIS (MITCHILL)

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(Received for publication, November 2, 1939)

The incubation of whitefish eggs occurs in nature at extremely low temperatures that fluctuate within a very narrow range. In Lake Erie, whitefish spawn in late November when decreasing water temperatures reach about 6°C. Soon the lake freezes over. Thereafter, whitefish eggs in ice-covered waters develop at temperatures only slightly above freezing for a protracted period of about 4 months of incubation. The peak of hatching occurs in the spring as the water approaches 6°C. Such development is in sharp contrast to that of the majority of fresh-water species which develop at considerably higher and more widely fluctuating temperatures during spring and summer. In the study herein reported, whitefish development was followed from fertilization to hatching at constant temperatures of 0°, 0.5°, 2°, 4°, 6°, 8°, 10°, and 12°C. The resulting data yield information regarding the normality and rate of development at these several temperatures. Certain accelerative effects of temperature have been treated statistically and presented graphically.

Materials and Methods[1]

For this study, approximately 120,000 whitefish eggs have been incubated in three annual series, during the winters of 1934–35; 1935–36; 1937–38. At the height of the spawning season each of these years, eggs were stripped from females taken alive from commercial gill nets in Lake Erie. The eggs were at once fertilized with milt from live males, covered with water, and handled with special care by an experienced fisherman. Received from the fishing boat, the eggs were at once transported in clean lake water cooled with floating ice to the refrigeration cabinets in the Zoology Department, Ohio State University, at Columbus. Transferred to incubation jars, the eggs were incubated thereafter at constant temperatures as indicated.

In each case, eggs were placed in these cabinets when in the one- or two-celled stage. In the first two series, the eggs were gradually shifted during the first 24 hour period

[1] The author wishes to thank Dr. T. H. Langlois, and Mr. E. L. Wickliff of the Ohio State Division of Conservation for their cooperation in providing whitefish eggs for this study.

into their respective temperature cabinets, and appropriate corrections have been made in the tables in calculating the incubation time of the first few cleavages. The third year it was found practicable to place the eggs immediately in their incubation temperatures.

The whitefish egg is non-floating and measures 3 mm. in diameter. A thousand eggs occupy about 30 cc. of the bottom of a water-filled cylinder, and a quart jar carries about 30,000 eggs. Cone-shaped, 500 cc. graduated wide-mouthed flasks were filled to from ⅛ to ½ full of eggs, and covered by at least an equal volume of filtered lake water. A stream of air was introduced into the bottom of the flask by a vertical glass tube attached either to an individual air pump or to an air-pressure line which delivered 5 pounds pressure at the main valve. Air thus bubbling into the flask at the bottom both aerated the eggs and caused them to be rolled over one another, an action which automatically scarified the egg shell surface and kept it translucent.

In tests made previous to 1934, eggs kept in finger-bowls and not so aerated and agitated were definitely retarded in development in comparison with controls. They gradually acquired opaque shells which often supported growths of *Saprolegnia* and *Vorticella*. Such shells eventually disintegrated and sloughed off their outer membrane. Within, there was a gradual necrosis of the embryo, usually beginning at the tail. All such unaerated eggs died at temperatures above 5°C., and no fish hatched at even 2°, a temperature close to the optimum for incubating aerated eggs.

In the present study, only data from eggs so aerated are recorded. There was no evidence of injury from excessive aeration or agitation.

Filtered water taken some considerable distance from shore in Lake Erie and transported in glass carboys was the only water used in the incubation jars. Various samples tested had a pH value of from 8.0 to 8.2. At various times, this Lake Erie water taken from jars containing incubating eggs was found to range from 7.8 to 8.4 in pH readings. Hall (1925) found that a pH of 7.8 is optimum for whitefish incubation in early stages and that the optimum is lowered as the embryo becomes older. The writer regards the O_2/CO_2 ratio and the hydrogen ion concentration to have been nearly optimum and quite uniform throughout these tests, in view of the abundant aeration provided.

Controlled Temperature Cabinets.—The flasks containing incubating eggs were kept in controlled temperature air cabinets, described by Peterson (1934). Briefly, the six insulated air cabinets, each with an inner storage space of 27 cubic feet, are cooled by brine flowing through coils in such a way as to maintain a cabinet temperature 1–2°C. below that desired. An insulated brine tank is kept at −4 to −5°C. through the action of an ammonia compressor of ⅜ ton capacity. In each air cabinet an electric fan forces a draught of air over the coils and throughout the cabinet proper. The coils are separated from the air chamber by an insulated panel.

The air of the cabinets is maintained at the desired temperature by the action of an American Instrument Company sealed mercury "Metastatic" thermoregulator, Type C, with a sensitivity of 0.02°C. which operates light bulbs through a supersensitive relay. The relay operates on 0.007 amperes at 6 volts. The air in the cabinets is thus maintained at the desired temperature with variations of not more than 0.1°C. A continuous temperature record is provided by a Foxboro temperature recorder attached to each cabinet.

The coldest cabinet was maintained, as stated above, at an air temperature of 0°C. Still water, of course, freezes at this temperature. It was possible, however, to prevent

ice formation in the incubation jars through agitation produced by air bubbling through the water. A slight temperature depression caused by a light bulb burning out or a sticking brine valve, or if an air pump failed to force air through the incubation jar, would permit freezing. Such difficulties were encountered in both of two series of eggs incubated at this temperature. Freezing resulted in high mortality, and the remaining eggs were thereafter discarded. Hence, the data for the 0° series are incomplete.

The Handling of Eggs.—Daily observations and records were made of mortality and stage of development attained. All dead eggs were removed each day from the incubation jars. To do this, the eggs were transferred to finger-bowls, which were stacked in the cabinet until examined. The finger-bowls, removed singly, were kept half submerged in prechilled water kept cold with floating ice. Dead eggs were removed as rapidly as possible with pipettes specially drawn.

For stage determination, it was found that twenty-five eggs constituted a valid sample, *i.e.* by chance, among twenty-five eggs would be found essentially the same proportion of all stages present as would be found in the entire lot of eggs. All stages found in the sample were recorded daily. The midpoint of time required for a given jar of eggs to attain a certain stage was designated as that time at which 50 per cent or more of the sample had attained that stage.

Types and proportions of abnormalities in the various series were also noted. A discussion of these types will be published elsewhere.

Description of Stages

Throughout this study, stages of development are referred to by numbers which correspond to the numbers of similar stages in the 803-stage Ohio State University series, collected at the Ohio State Fish Hatchery at Put-in-Bay, Ohio, during the winter of 1926–27, and described by me in previous papers (Price, 1934 and 1935). Supplementing that description made solely from preserved material, certain features peculiar to living embryos have been used in the present study as diagnostic features.

It is noteworthy that two criteria often used for designating stages were found unreliable in this study. These are (1) embryo length and (2) the number of somites. Embryos incubated at the higher temperatures were uniformly shorter and smaller at a given stage of development otherwise than were embryos incubated in the lower range of temperature. At the higher temperatures likewise, many abnormal embryos were distorted in the midbody and tail regions, rendering a somite count futile. Embryo length and somite count as criteria were therefore discarded in favor of a combination of both structural and physiological features which proved to be more satisfactory.

Pigment appears in the eye, in the blood, and in chromatophores in a definite sequence. In just as precise a manner, the circulatory system developed in a consistent pattern, and the visible extent of the vitelline circulation seemingly varied within extremely narrow limits from one em-

TABLE I

Series of Stages Used in the Determination of the Degree of Development of Live Whitefish Embryos

Stage	Diagnostic characteristics
1-cell	Unsegmented blastodisc
2-cell	2-celled "
8-cell	8-celled "
16-cell	16-celled "
32-cell	32-celled "
No. 8 O.S.U.	Blastodisc 4 cells deep. Early periblastic ridge.
16 "	Blastodisc 8–10 cells deep. Blastomeres small.
32 "	Formation of germ ring and sub-germinal cavity.
48 "	Blastoderm envelops upper ⅛ of yolk.
64 "	Blastoderm envelops upper ½ of yolk.
80 "	Blastoderm envelops upper ⅜ of yolk. Primary germ layers established.
96 "	Large yolk plug stage. Formation of embryonic streak.
112 "	Small yolk plug stage. Optic primordia appear.
128 "	Closure of blastopore. Three primary cerebral vesicles developed. Notochord extends forward to level of mesencephalon.
144 "	Embryo extends ½ distance around curvature of yolk, without torsion. Auditory anlagen visible.
160 "	Increased size of brain and optic vesicles. Two layered optic cup, with choroid fissure and lens primordium. Nasal pit anlagen appear.
176 "	Progressive development of features listed above.
192 "	Fourth ventricle visible. Optic stalk present. Tail begins to be undercut by tail fold.
224 "	First heart beat. Embryo ⅜ way around yolk. First anterior branchial pouch has perforated. Prominent fourth ventricle.
272 "	First curve of tail. First movements, spasmodic in mid-body regions.
320 "	Dorsal aortae traceable into tail region. First eye pigment visible.
368 "	First color of blood, detectable in heart region.
400 "	Embryo complete circle on yolk in series incubated at low temperatures. Heart U-shaped. Vitelline veins large and filled with erythrocytes. Semi-circular canals developing. Pectoral fins definitely raised above surface of lateral somatopleure. First eye pigment readily apparent.
448 "	Circulation very evident in extra-embryonic area. Eye pigment darker.
496 "	Pectoral fins definitely formed. Length slightly less than 8 mm. in case of normal embryos.
528 "	Circulation very clear throughout tail, body, and on sides of yolk. Hatching enzyme glands visible in throat region. Yolk vessels at periphery of splanchnocoele form single continuous loop from vitelline artery to vein. Two chambered heart.
592 "	Circulation in yolk vessels visible, in branch of vitelline vein lateral to heart on yolk. Spasmodic twisting of entire body, rotating yolk sac as it does so. No movements of pectoral fins. Estimated length of normal embryos 8.5 mm.

TABLE I—*Concluded*

Stage	Diagnostic characteristics
No. 632 O.S.U.	Three semi-circular canals clearly visible through shell. Yolk with single large oil globule. Circulation in vitelline veins traceable from below oil globule. Eyes black. Caudal lobe on tail. No movement in pectoral fins. Embryo occasionally goes through spasmodic and violent twisting of entire body, changing the flexure from left to right and *vice versa*, in some cases moving entire yolk sac.
680 "	First evidence noted of pectoral fin movement. Pectoral fin spearhead shaped from dorsal view. Stellate chromatophores in double row along entire length of body. Heart beneath body.
776 "	Constant fin flutter stage. Pectoral fins in a state of rapid fluttering or twitching.
800 "	Stellate chromatophores appear for first time on head. Embryo extremely active.
	Hatching stage.

bryo to another of the same stage. The time of the initiation of characteristic movements, first spasmodic and general in the midbody region, followed by tail movements, then general body-twisting movements, and finally fin and eye movements all follow in as strict a chronological sequence as the development of morphological features. All these, taken together, form the basis for the designation of stages, in Table I.

RESULTS

I. Range of Temperature Tolerance.—Data given below (Tables II to IV) show that whitefish hatched alive at all temperatures tested between 0.5°C. and 10°C. Although certain development occurred at the extreme temperatures of 0°C. and 12°C., no fish hatched at these extremes. The effective range of temperatures is therefore limited to from 0.5–10°C., through which hatching occurred. The proportion of normal live fish hatched at intermediate temperatures suggests a range of 0.5–6°C. for normal development with an optimum at 0.5°C.. A more complete statement follows.

The freezing of water at 0°C. imposes a natural limitation to development below that point. When freezing could be prevented at 0°C., development occurred although it was extremely retarded. (See Table IV.) Various tests showed that, upon being frozen, eggs in early cleavage stages underwent a disruption of the numerous scattered oil globules beneath the blastodisc. These globules then either coalesced within the yolk into one or two large globules, or were cast off into the perivitelline space. The blastodisc simultaneously became distorted and shortly thereafter opaque. If freezing were prevented at 0°C. until after gastrulation, it failed to cause any distortion visible externally, but general necrosis soon became evident.

In two years' tests, the writer reared eggs at 0°C. without freezing to stages 224 and 272 respectively, beyond gastrulation (stage 128). However, mortality in each case had reached at least 95 per cent. Whether eggs could be induced to hatch at this extreme temperature is seriously open to question. These results strongly indicate that 0°C. is the lowest temperature at which whitefish eggs will develop; *i.e.*, the physiological zero.

The *highest* temperature tested at which development occurred was 12°C. (1935–36 series). Development proceeded at a consistent rate, although mortality was extremely high from the beginning. In later stages, all surviving embryos were noticeably abnormal, the majority of them having short, twisted tails and distorted heads. A few hatched dead, but none

TABLE II

Mortality, Hatchability, and Abnormality of Whitefish Embryos Incubated at Constant Temperatures. 1934–35 Series

	Incubation temperatures					
	0.5	2.0	4	6	8	10
	°C.	°C.	°C.	°C.	°C.	°C.
Mortality, per cent						
(*a*) Prior to hatching stage	26.25	38.0	40.0	27.5	34.4	63.0
(*b*) During hatching stage	1.08	4.0	1.4	14.0	46.8	36.4
(*c*) Total	27.33	42.0	41.4	41.5	81.2	99.4
Eggs hatched alive, *per cent*	72.67	58.0	58.6	58.5	18.8	0.6
Estimated embryos alive at hatching stage that were *abnormal, per cent*	0	0	1	10	25	50

hatched alive. This temperature is therefore regarded as beyond the range of tolerance of the whitefish egg for effective incubation and survival.

The comparative survival and hatchability of whitefish eggs incubated throughout the effective temperature range of 0.5–10°C. is summarized in Table II from the 1934–35 series.

From these results, eggs incubated at 10°C. are seen to have a high mortality, 99 per cent total, and low hatchability, less than 1 per cent. The estimate of 50 per cent of the embryos alive at the hatching stage being abnormal is probably too low. In the other two series, the percentage of abnormals produced at this temperature was 95–100 per cent. At best, a temperature of 10°C. is extremely adverse to whitefish embryonic development.

The next lower 8° temperature, while less unfavorable, still produced a significant proportion of abnormal embryos, a high mortality, and a low hatch.

Temperatures from 2–6° yielded a quite uniform result in the percentage of incubated eggs hatched (58 per cent). Differences in the mortality records occur in the higher percentage of eggs dying at the time of hatching at 6°, although the total mortality for this series at 6° is approximately the same as for the 4° and 2° series. The 10 per cent of abnormal fishes alive at hatching time indicate 6° as the *maximum* temperature at which normal development characteristically occurs. Day old fishes hatched at this temperature swim vigorously in a tank of water, whereas those hatched at higher temperatures exhibit only occasional movements, lying most of the time quietly on the bottom of the tank.

The most favorable results were obtained in these tests in the 0.5°C. series. The relative proportion of fishes hatched, the lower mortality throughout, and the absence of abnormal fishes produced support this conclusion. In this connection, the typical lengths of newly hatched fishes incubated at various temperatures are interesting.

TABLE III

Lengths in Millimeters of Newly Hatched Whitefish, Incubated at Various Constant Temperatures (1934–35)

10°	8°	6°	4°	2°	0.5°
8–9.5		11–12		11–13	12–14

These results in length of hatched whitefish correspond to those obtained both by Hall (1925) at these same temperatures so far as tested, and at the lower temperatures with those typically secured at the Put-in-Bay, Ohio hatchery on Lake Erie. The above data seem to justify the conclusion that 0.5°C. is the *optimum* or at least very close to the optimum temperature of incubation of the whitefish egg.

From the above one may readily perceive that the whitefish is an unusually clear cut case of a species being precisely adjusted during its embryonic development, to a very narrow range of an environmental factor, 0.5–6°C. temperature, with the optimum for the species being extremely close not only to the limit for the medium (freezing point of water) but also to the physiological zero for many processes. Such a low temperature range obviously limits the occurrence and continued survival of the species to waters which maintain a winter time temperature close to the freezing point. Actually, whitefish do occur both in North America and in Europe, principally in lakes which, like the Great Lakes, are subject to continuous freezing in winter.

TABLE IV

Incubation of Whitefish Eggs in Three Annual Series; A = 1934–35 Series; B = 1935–36 Series; C = 1937–38 Series. Av. = Average Time in Days for the Three Series. K = Time Calculated from Equation

$$T = \frac{M}{A^{\frac{t}{1}}}\Bigg\}\begin{matrix}0^\circ\\6^\circ\end{matrix};\ T = \frac{M}{A^{\frac{t}{2}}}\Bigg\}\begin{matrix}6^\circ\\12^\circ\end{matrix}$$

Stage No.		Temperature of incubation							
		0°	0.5°	2°	4°	6°	8°	10°	12°
1-cell		0	0	0	0	0	0	0	0
2-cells	*A*	—		—					
	B	0.83		0.5					
	C	0.75	—	0.5	—	—	—	—	—
	Av.	0.79		0.5					
4-cells	*A*	—		—	—	—			
	B	1.41		0.83	—	0.5			
	C	—	—	—	0.75	0.5	—	—	—
	Av.	1.41		0.83	0.75	0.5			
8-cells	*A*	—		—		—	—		
	B	1.83		1.41		0.83	0.5		
	C	—	—	—	—	0.83	0.75	—	—
	Av.	1.83		1.41		0.83	0.63		
16-cells	*A*			—				—	
	B			1.83				0.5	
	C	—	—	—	—	—	—	0.75	—
	Av.			1.83				0.63	
32-cells	*A*	—					—		—
	B	2.83					0.83		0.5
	C	—	—	—	—	—	—	—	—
	Av.	2.83					0.83		0.5
Stage No. 8	*A*	—	3.4	2.83	2.1	—		—	—
	B	3.54	—	2.83	—	1.41		1.0	0.83
	C	3.87	—	—	—	—	—	—	—
	Av.	3.71	3.4	2.83	2.1	1.41	—	1.0	0.83
	K	3.82	3.52	2.77	2.01	1.43	1.18	0.99	0.83
16	*A*	—	4.4	3.83	3.6	2.0	1.83	1.6	—
	B	4.41	—	3.45	—	2.0	1.83	1.6	1.41
	C	6.5	—	4.5	4.0	3.8	—	—	—
	Av.	5.45	4.4	3.92	3.8	2.6	1.83	1.6	1.41
	K	5.34	5.05	4.26	3.4	2.58	2.0	1.64	1.35

TABLE IV—*Continued*

Stage No.		Temperature of incubation							
		0°	0.5°	2°	4°	6°	8°	10°	12°
32	*A*	—		7.33	6.0	3.75	3.3	2.5	—
	B	8.5		7.5	—	4.41	3.58	2.83	1.83
	C	12.0		8.5	5.58	5.0	—	—	—
	Av.	10.25	—	7.78	5.59	4.39	3.44	2.66	1.83
	K	10.24	9.53	7.68	5.76	4.4	3.38	2.54	1.9
64	*A*	—	17.4	13.83	10.6	6.25	5.0	4.35	—
	B	18.6	—	14.0	—	7.0	5.5	4.6	4.4
	C	21.6	—	15.0	12.5	10.0	—	—	—
	Av.	20.1	17.4	14.28	11.6	7.75	5.25	4.47	4.4
	K	20.08	18.6	14.78	10.87	7.5	5.84	4.84	4.01
128	*A*	—	28.4	23.33	15.6	9.62	7.3	6.6	—
	B	28.5	—	18.6	—	—	—	6.46	5.41
	C	(33.0)	—	23.6	15.5	13.0	9.16	—	—
	Av.	30.75	28.4	21.84	15.55	11.31	8.23	6.53	5.41
	K	30.60	28.15	21.91	15.68	11.05	8.55	6.70	5.25
224	*A*	—	(46.5)	42.83	23.8	16.55	(12.17)	—	—
	B	43.38	—	26.46	—	15.41	—	9.41	(8.0)
	C	55.5	—	38.5	23.75	19.83	15.3	10.5	—
	Av.	49.44	(46.5)	35.93	23.77	17.26	13.73	9.95	8.0
	K	49.92	45.65	34.93	24.44	17.25	13.66	10.28	7.9
272	*A*	—	54.5	48.0	27.6	19.3	13.67	10.0	—
	B	—	—	36.7	—	20.5	—	10.41	8.5
	C	60.5	—	41.5	(26.75)	(20.75)	(15.78)	(11.3)	—
	Av.	60.5	54.5	42.06	27.17	20.18	14.72	10.57	8.5
	K	60.15	54.79	41.43	28.52	19.7	14.80	11.05	8.24
400	*A*		63.5	60.3	33.6	22.17	17.5	13.6	—
	B		—	39.41	—	23.5	—	12.41	10.91
	C		—	(53.5)	34.75	—	18.33	13.08	—
	Av.		63.5	51.07	34.17	22.83	17.91	13.03	10.91
	K		69.09	51.08	34.15	22.75	17.63	13.68	10.62
448	*A*		71.5	67.0	37.6	24.75	20.8	15.6	—
	B		—	43.38	—	25.5	—	13.41	11.41
	C		—	55.5	38.5	25.75	20.16	—	—
	Av.		71.5	55.96	38.05	25.33	20.48	14.5	11.41
	K		75.6	56.16	37.78	25.6	19.64	14.93	11.35
528	*A*		78.7	73.0	43.6	27.25	24.34	—	—
	B		—	54.5	—	27.5	—	15.41	13.41
	C		—	60.5	42.5	27.75	(22.25)	16.25	—
	Av.		78.7	62.66	43.05	27.5	23.29	15.84	13.41
	K		86.37	63.43	42.02	27.98	21.81	16.92	13.12

TABLE IV—*Concluded*

Stage No.		Temperature of incubation							
		0°	0.5°	2°	4°	6°	8°	10°	12°
632	*A*		89.0	82.0	(52.6)	—	27.8	21.1	—
	B		—	—	—	30.5	—	17.4	15.41
	C		—	65.5	48.0	(37.0)	26.25	18.53	—
	Av.		89.0	73.75	50.3	33.75	27.02	19.01	15.41
	K		99.14	73.95	50.03	33.98	26.02	19.86	15.15
776	*A*		113.5	100.0	67.6	38.17	32.3	25.6	
	B		—	—	—	40.83	—	26.46	
	C		—	84.5	59.0	44.5	33.25	24.25	
	Av.		113.5	92.25	63.3	41.16	32.77	25.44	
	K		126.0	93.08	62.18	41.43	32.5	25.55	
Hatching	*A*		141.0	133.0	83.6	57.25	40.3	29.6	
	B		—	—	—	56.5	—	—	
	C		—	109.0	77.0	62.0	—	—	
	Av.		141.0	121.0	80.3	58.58	40.3	29.6	
	K		156.35	119.1	82.87	57.8	41.19	29.28	

II. Rate of Development.—The time in days required for whitefish eggs to attain the various stages described in Table I is listed in Table IV. Average values for the 3 years' tests are given. In some instances, the variation between different year groups was considerable. In no case, however, was there overlapping for the different year groups between successive stages in a given series, or in the time required for attaining the same stage at different temperatures. The variation is such, however, that the mean values of 3 years' tests are more consistent and probably more representative than are any one year's values alone.

One sees from Table IV that in whitefish eggs, the length of the developmental period up to any given stage varies inversely with the temperature, and that the spread in days between successive temperature series increases progressively with development. The relative difference in time between various temperature series remains, however, about the same. For instance, early development through gastrulation is complete by stage 128. This period increases from 6.53 days at 10°C. to 28.4 days at 0.5°C., an increase of 4.3 times. Likewise the total incubation period to hatching increases from 29.6 days to 141 days at these same temperatures, an increase of 4.7 times. Relatively therefore the differences are approximately the same.

In round numbers, a rise of 4°C. either halves the time or doubles the rate of attaining a given stage, and Q_{10} values fluctuate around 5.0 as a mean.

Chart 1 has been drawn by plotting the average observed time values of Table IV. The comparative slope of the lines indicates the relative rate of development at various temperatures. In general, the rate, $\frac{1}{\text{days}}$, of development is directly proportional to temperature.

No attempt has been made to smooth these lines. Some of the variations from the rectilinear are undoubtedly due to practical difficulties in determining end-points. However, the tendency of these lines to undulate somewhat regularly can scarcely be accounted for on this basis. They

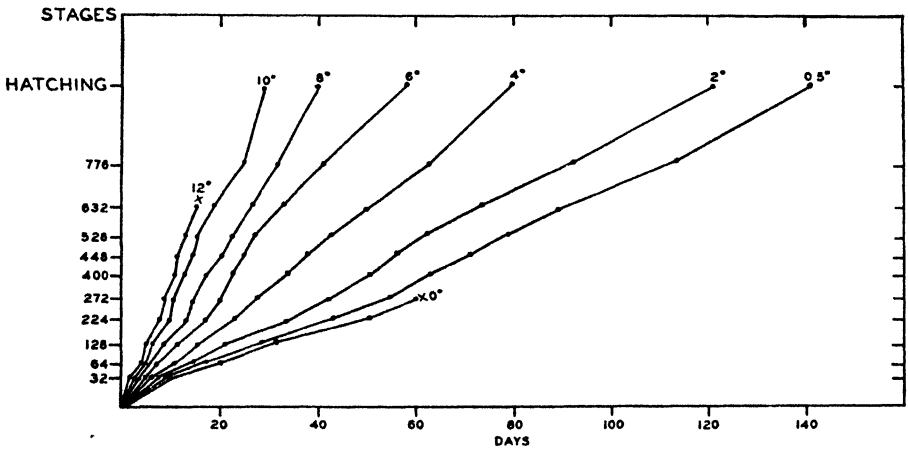


CHART 1. Velocity of development at different constant temperatures. Average time in days for each temperature for each stage.

somewhat suggest the rhythmic variation in growth rate of the chick (Schmalhausen *et al.*). Treated from another point of view, variations in rate during certain developmental periods are discussed at the end of this paper.

III. Determination of Thermal Increments.—Whether a single degree difference in incubation temperature results in the same proportionate amount of change in rate of development throughout the temperature range can be shown by the calculation of thermal increments or temperature characteristics.

For this purpose, stage 272 was arbitrarily selected as a test case. In the lower portion of Chart 2, the average observed number of days of incubation up to stage 272, as listed in Table IV, is plotted against temperatures. The logarithms of time (log T) plotted in the upper portion of this

chart rectify the natural number curves. By applying the method of least squares (Snedecor *et al.*) the two straight lines shown were fitted to these points (log T; C°). The equations used are:

$$T = \frac{M}{A_1^t}\Bigg\}\begin{matrix}0^\circ\\6^\circ\end{matrix}; \qquad T = \frac{M}{A_2^t}\Bigg\}\begin{matrix}6^\circ\\12^\circ\end{matrix} \tag{1}$$

$$\log T = \log M - t \log A \tag{2}$$

in which the time (T) to attain a given stage is inversely proportional to the constant (A) raised to an exponent, temperature (t). The constant

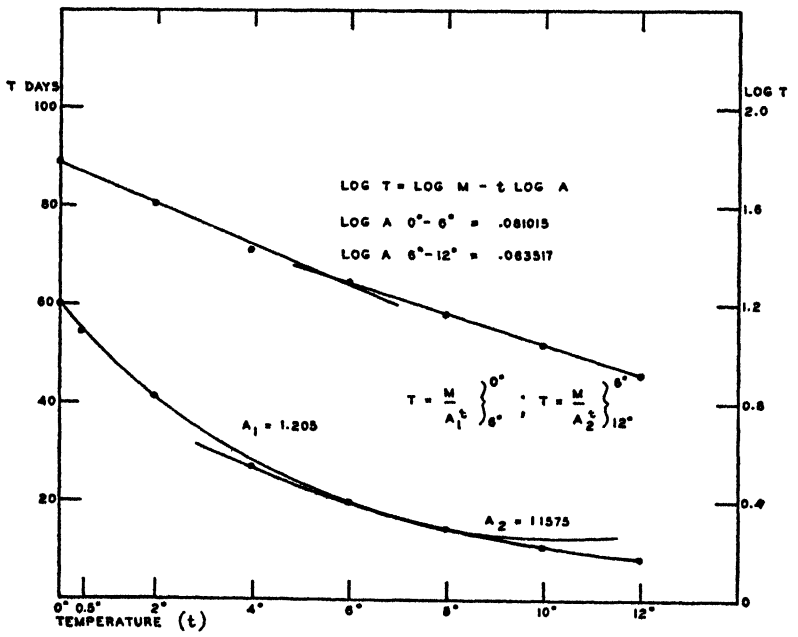


CHART 2. Incubation time as a function of temperature; stage 272

(M) is the Y-intercept at 0° ordinate. Since, within the temperature range given, values of A are constant for a given stage, the term A in equation (1) above serves as a temperature characteristic.

In fitting various curves to the points on these semi-log graphs, goodness of fit was measured by calculation of the standard error of estimate. A single straight line of equation (2) fitted to the semi-log curve from 0–12° had a standard error of estimate of 0.028. The combination of two straight lines as drawn on the semi-log graph has a standard error of estimate of only 0.0138, a reduction of more than half. Thus by resolving the semi-log curve for this stage into two straight lines, instead of one, estimated values

and the curves drawn from them more nearly agree with mean observed values. These lines intersect one another at the 6° locus. In equations (1) and (2), values of A derived from log A measure the slopes of these lines. They have one value below 6°C. and another value above it. Values of A regarded as temperature characteristics, express the relationship between time and temperature. In stage 272, $A_1 = 1.205$ between 0° and 6°, $A_2 = 1.1575$ between 6° and 12°. Equation (1) now becomes

$$T = \frac{M}{1.205^t}\Bigg\}\begin{matrix}0^\circ\\6^\circ\end{matrix}; \qquad T = \frac{M}{1.1575^t}\Bigg\}\begin{matrix}6^\circ\\12^\circ\end{matrix} \tag{3}$$

This is to say that below 6°, increasing the temperature 1° increases the speed of development 1.205 times; above 6°, increasing the temperature 1° increases the velocity 1.1575 times.

From the above result, 6° is a critical temperature, above and below which the temperature characteristics differ. In this particular case in question, curves which intersect just below 6° fit the observed points closer than do other curves drawn to intersect just above 6°, but neither differ significantly in their standard errors of estimate from that of the curves drawn intersecting at the 6° locus. The critical point in the temperature range for this stage then is 6° or slightly below.

Bělehrádek has studied time/temperature relations in a variety of circumstances, in which he has applied the empirical equations

$$y = \frac{a}{x^b} \tag{4}$$

$$\log y = \log a - b \log x \tag{5}$$

Here time, (y) is expressed as the reciprocal of the temperature, (x), raised to its exponent, b, the latter being regarded as a temperature characteristic. To express this relationship graphically, log y values are plotted against log x values. The constant a expresses the time at ± 1°, since log 1.00 is zero. The slope of the resulting straight line curve or curves expresses the value of b. For stage 272, a single straight line fitted to the log y/log x points yields a standard error of estimate of 0.063. Two lines intersecting at the 6° locus and fitted by the method of least squares yield a standard error of estimate of 0.0094, a reduction of 6.7 times over the error in the single line curve. In this case, equation (4) becomes

$$y = \frac{a}{x^{0.0644}}\Bigg\}\begin{matrix}1^\circ\\6^\circ\end{matrix}; \qquad y = \frac{a}{x^{1.265}}\Bigg\}\begin{matrix}6^\circ\\12^\circ\end{matrix} \tag{6}$$

From the above it is seen that equation (6) with a standard error of estimate

of 0.0094 can be made to fit the data just as closely over its range as does equation (3) with a standard error of estimate of 0.0138. Both equations indicate that 6° is a critical temperature. However, b values of equations (4) and (6) represent acceleration in log rate with reference to that rate at the base temperature of 1°. Obviously b in these equations is an exponent, expressing the power to which the temperature is raised as a function of time. It is not readily susceptible to a direct calculation in terms of natural numbers, as is the case with the natural number values of A in equations (1) and (3). This fact favors the use of the latter in deriving a temperature characteristic from an exponential equation. Further, equation (6) does not hold for the 0° temperature, and at 0.5°, there is a wide difference between calculated and observed values. For these reasons, equation (6) is discarded in this analysis in favor of equations (1) and (2) in expressing the time/temperature relations in the incubation of the whitefish.

Returning then to Chart 2, the lines as drawn express the time/temperature relations applying to stage 272. From either the chart directly or from the values of A given, it is seen that (1) the time required to attain stage 272 throughout the entire range from 0–12° through which whitefish eggs will develop is expressed as a combination of two negative exponential curves; (2) the mid-point in the temperature range, 6°, is a critical temperature. It will be recalled here that 6°C. is the temperature below which normal development typically occurs; above it, progressively abnormal development occurs; (3) below this critical temperature, the temperature characteristic, $A_1 = 1.205$, applies, meaning that for every degree rise in incubation temperature, the time required to attain this stage is decreased 1.205 times. Above 6°, the temperature characteristic, $A_1 = 1.1575$, applies, expressing a proportionate decrease in time per degree rise in incubation temperature.

Treating the data for the other stages studied, in a similar fashion, using the method of least squares for smoothing curves and calculating the values of A, results similar to the above were obtained. Two curves which intersect at the 6° locus give a closer fit to the data than does a single curve without such a break. The closeness of fit was satisfactory except at the 0.5° locus in later stages, where observed values were definitely lower than those calculated.

Calculated A values for the various stages are displayed in Table V. A values for temperatures below 6°C. have a mean of 1.193, while those for temperatures above 6°C. have a mean of 1.135. That these mean A values are significantly different is shown by the fact that their difference is more than five times the standard error of their difference.

Smoothed curves which show the time calculated from the above for whitefish eggs to attain certain significant stages are presented in Chart 3. Points indicated represent the average observed time of incubation at each temperature.

The estimated numbers of days for other stages based on similar calculations are listed in Table IV.

TABLE V

Temperature Characteristics of Whitefish Egg Incubation above or below the Critical Temperature, 6°C. A Values Express Acceleration Per Degree Rise in Temperature, from Fertilization to the Attainment of the Stage Given. For Stage Descriptions, See Table I

To stage	$A_{0-6°}$ values	$A_{6-12°}$ values
2-cell	1.257	—
4-cell	1.173	—
8-cell	1.141	—
8	1.173	1.092
16	1.119	1.103
32	1.154	1.155
64	1.165	1.098
128	1.181	1.130
224	1.195	1.140
272	1.205	1.157
400	1.223	1.135
448	1.219	1.146
528	1.228	1.135
632	1.215	1.144
776	1.223	1.128
Hatching	1.199	1.186
Mean =	1.19266	1.13486
Standard error of mean =	∓.00865	∓.00687
Difference between means =	5.235 × (standard error of their difference)	

The instantaneous responses to temperature during various phases of embryonic development are revealed when thermal increments are calculated for each phase independently. Thermal increments in terms of both the A values of the exponential equation above and μ values of Arrhenius' equation, as used by Crozier and others, have been determined for the following phases: From 1-cell stage to the 8-cell stage, designated as *early cleavage*; from 8-cell to stage 8, designated as *mid-cleavage*; stages 8–32, *late cleavage*; stages 32–128, *gastrulation*; stages 128–400, *organogenesis*; stages 400–776, subsequent *growth* to pre-hatching stage; stage 776 to mid-

hatching date, *hatching* period; fertilization to hatching, *total* incubation period.

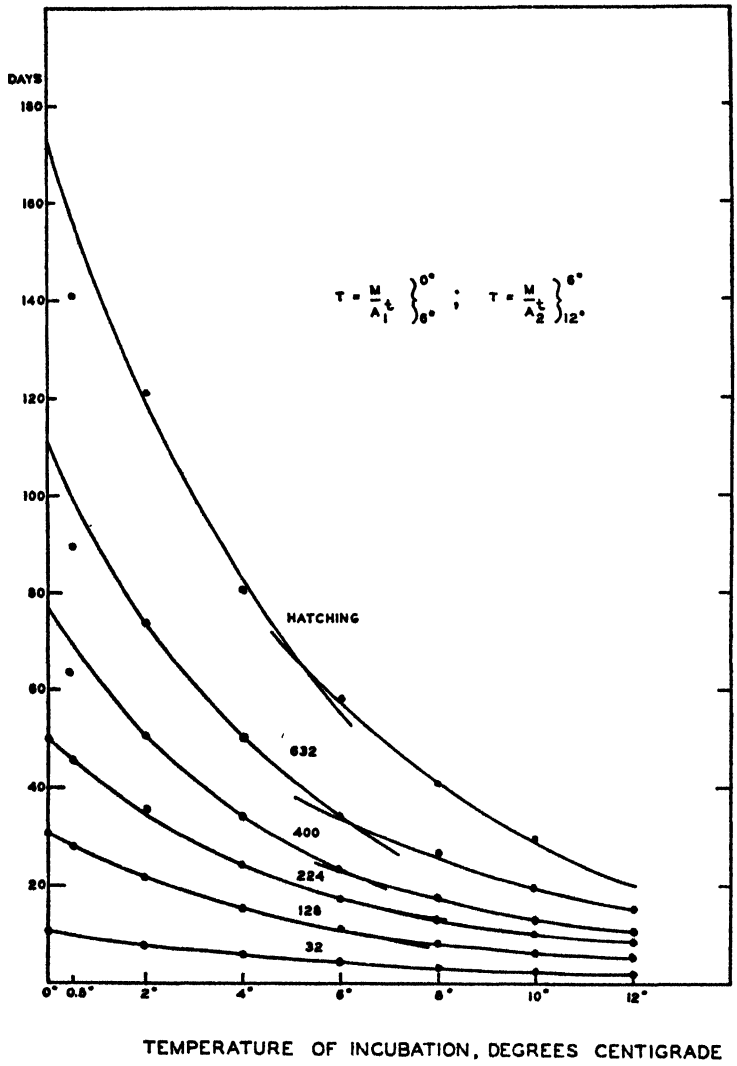
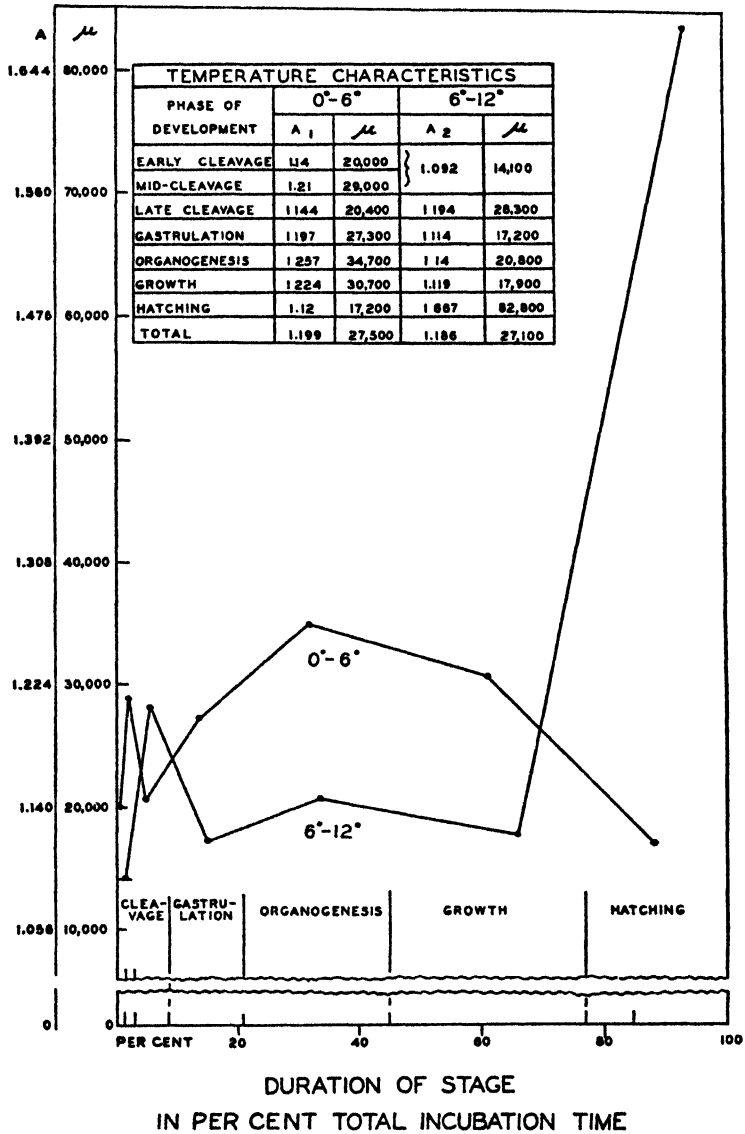


CHART 3. Length of incubation period of whitefish eggs at temperatures given, to attain certain selected stages.

Stage 32, end of cleavage	Stage 400, complete circle
Stage 128, closure of blastopore	Stage 632, growth
Stage 224, primary organogenesis	Hatching stage

The A and μ values for these several developmental phases are listed and plotted in Chart 4. Along the abscissae, the space occupied by each phase

shows its duration in per cent of the total incubation time. The proportionate part that each phase occupies of the whole is listed in Table VI.



TEMPERATURE CHARACTERISTICS				
PHASE OF DEVELOPMENT	0°-6°		6°-12°	
	A_1	μ	A_2	μ
EARLY CLEAVAGE	1.14	20,000	1.092	14,100
MID-CLEAVAGE	1.21	29,000		
LATE CLEAVAGE	1.144	20,400	1.194	28,300
GASTRULATION	1.197	27,300	1.114	17,200
ORGANOGENESIS	1.257	34,700	1.14	20,800
GROWTH	1.224	30,700	1.119	17,900
HATCHING	1.12	17,200	1.667	82,800
TOTAL	1.199	27,500	1.186	27,100

CHART 4. Instantaneous temperature characteristics for various periods of whitefish development.

Table VI shows that proportionate lengths of *pre-hatching* phases of development are not greatly altered by the temperature of incubation.

The mean length of the *hatching* period above 6°, however, is only slightly more than half (57.5 per cent) its mean length below that point. Obviously the hatching process is affected by temperature differently in the two portions of this range.

An especially interesting outcome shown in Chart 4, is the correspondence in fluctuations in A and μ values for these phases of development, since they describe the time/temperature relationship in different terms. As mentioned above, A values are a measure directly of the proportionate amount by which time of development is increased per degree Centigrade drop in temperature, or by which velocity is increased per degree rise in temperature, within a given range. Their utility is in interpolation. On the other hand, μ values have been interpreted to indicate the gram molecular energy of activation of the catalyst; *i.e.*, the "critical increment of the active substance" controlling the reaction. Their utility is in identifying, presumably, the character of the controlling reactions.

TABLE VI

Relative Duration of Various Stages

	0–6°	6–12°
	per cent	*per cent*
1-cell to 8-cell	1.25	3.24
8-cells to stage 8	1.16	
Stages 8– 32	4.37	5.77
Stages 32–128	12.00	13.92
Stages 128–400	23.37	23.88
Stages 400–776	34.65	39.84
Stages 776–hatching	23.20	13.35
	100.00	100.00

On either basis, the curves show similar trends, and that the whitefish embryo throughout its range of incubation temperatures responds in a cyclic manner to temperature differences. Peaks occur in both curves during the periods of cleavage and of organ formation. The peak during cleavage of the 6–12° curve is relatively 1.5 times higher above its origin than is the 0–6° curve. This is to say that the rate of change per degree Centigrade during cleavage is approximately 1.5 times greater in the higher temperature range than it is in the lower range at its maximal point. The sharpness of the peaks here may indicate a relative instability of the interacting factors. That the egg is highly sensitive to temperature effects during cleavage is borne out by the extreme mortality of eggs subject to adverse temperatures during this phase.

Beyond these points, the curves indicate that the rate of change per degree temperature is uniformly lower for the higher temperatures. The less acute and somewhat more regular angles of the curves may indicate an accumulative effect with age as to the organism's responsiveness to temperature.

One might be permitted the suggestion that up to the pre-hatching stage these curves represent the relative interaction to temperature of two separate sets of embryonic processes. The first set of processes consist of those which give a maximal response by way of rate of change to temperature differences early in development; *i.e.*, prior to gastrulation. The second set of processes are such that they give a maximal response during later stages.

The duration of the hatching period is primarily dependent upon processes that effect a rupture of the egg-shell membranes. From both observation and experiments, the hatching of whitefish eggs results from a combination of mechanical movements of the fish within the shell and the action of hatching enzymes. Temperature effects upon the rate of action of these factors may or may not be predictably similar to effects of temperature upon the speed of embryonic processes.

In Chart 4, the downward slope of the 0–6° line during the hatching period is directly opposed by the sharply rising 6–12° line. The magnitude of both the A and μ values during the hatching period in the upper temperature range indicates that hatching is probably controlled above 6° by factors unique to that process and not significant at temperatures below 6°.

The μ value of 82,800 does not at all correspond to such values for embryonic processes, as seen from the chart on the graph. Assuming that one or more catalytic agents are operating here as hatching enzymes, such enzymes must be extremely sensitive to temperature differences. This is supported by the fact that the hatching period lasts relatively only 57.5 per cent as long above 6° as it does below 6°. Likewise, a high mortality of embryos at the hatching period at high temperatures suggests an imbalance between interacting factors as affected by such temperatures.

SUMMARY

1. Whitefish eggs incubated in aerated lake water at controlled temperatures of 0°, 0.5°, 2°, 4°, 6°, 8°, 10°, and 12°C., failed to hatch at either 0° or 12°C. 0.6 per cent hatched alive at 10°C., 72.67 per cent hatched alive at 0.5°C., and an intermediate proportion hatched at intermediate temperatures.

2. The percentage of abnormal embryos which developed to the hatching stage varied directly with temperature between 4° and 12°, all embryos

being abnormal at 12°C.; but none were abnormal at either 0.5°, or 2°C. Normal development predominated from 0.5 to 6°C. The highest proportion of embryos to hatch alive was 72.67 per cent at 0.5°C., which is, hence, the optimum temperature.

3. Total incubation time ranged from 29.6 days at 10°C. to 141 days at 0.5°C.

4. The time (T) required to attain any given stage of development is expressed in equations

$$T = \frac{M}{A_1^t}\Big\}\begin{matrix}0^\circ\\6^\circ\end{matrix}; \qquad T = \frac{M}{A_2^t}\Big\}\begin{matrix}6^\circ\\12^\circ\end{matrix}$$

where temperature, t, is a negative exponent of the constant, A, whose value differs above or below 6°C., a critical temperature. Values of A above 6° fluctuate about 1.13; those of A below 6° fluctuate about 1.19 as a mean.

5. Applying Arrhenius' equation μ values for the total incubation period are 27,500 below 6° and 27,100 above it.

6. The relative magnitude of A values of the exponential equation and μ values of Arrhenius' equation show corresponding changes from one developmental period to another.

7. When plotted, thermal increments show cyclic variations, with maxima during periods of cleavage and of organogenesis. These may indicate the interaction of two separate sets of embryonic processes, which give a maximal response to temperature differences during these two separate periods.

8. Above 6°, μ values during the hatching process are distinct from those of developmental stages and are regarded as being due to the action of hatching enzymes.

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THE PIGMENT-PROTEIN COMPOUND IN PHOTOSYNTHETIC BACTERIA

I. The Extraction and Properties of Photosynthin

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INTRODUCTION

It has been known since Herlitzka's work in 1912 that it is possible to prepare colloidal solutions of chlorophyll by grinding plant tissue. Lubimenko (1927) found that some leaves (*Funkia* and *Aspidistra* genuses) gave on extraction with water clear stable green solutions which contained all the chlorophyll of the original leaf in combination with a protein. Other leaves gave extracts which contained water soluble protein-combined chlorophyll but which were not stable, while still a third group of plants had the chlorophyll attached to an insoluble protein. He showed that the protein-chlorophyll solutions had the same absorption bands as the living leaf. Mestre presented additional evidence that indicated the occurrence of chlorophyll-protein compounds in green plants. Wald has measured the absorption curves and studied the protein properties of such solutions. Stoll and Wiedermann have made a careful study of the methods of preparation and the properties of these compounds, and Smith has published an absorption curve and preliminary centrifugal studies of the protein-chlorophyll solution extracted with digitalin from spinach indicating a molecular weight of over 70,000 in solution with digitalin. Incidental to the study of plant virus proteins Price and Wyckoff and Loring, Osborn, and Wyckoff have prepared pure solutions showing sharp sedimentation boundaries of the chlorophyll-protein compounds from peas and beans. Sedimentation constants of 77×10^{-13}, 117×10^{-13}, and 54×10^{-13} were found.

These studies all tend to show that in the living leaf chlorophyll occurs in combination with a protein much in the same way that hemin is attached to a protein forming hemoglobin. The protein apparently varies from species to species perhaps even more than does globin from different species of animals. As Stoll and Wiedermann as well as Smith have pointed out,

an analogy may also be drawn to the respiratory enzymes which likewise contain a prosthetic group attached to a protein. The chlorophyll-protein may be regarded as an enzyme of photosynthesis. Eisler and Portheim prepared a chlorophyll-protein compound by adding an alcoholic chlorophyll solution to serum diluted in water and to water extracts of colorless plants. Dolk and van Veen also have made such solutions but do not confirm the other workers' observations of an O_2 evolution from them in the light.

Lubimenko (1921) suggested the probability of the photosynthetic bacteria having their pigments in the form of a compound with a protein and Wurmser, Lévy, and Teissier prepared a water soluble protein pigment compound from a species of purple bacteria, *Chromatium*, and gave its absorption spectrum in the visible range. Short notes on the present work have appeared (French, 1938 *a* and *b*). The importance of the study of means of preparation of these solutions from photosynthetic organisms and the investigation of their properties lies in the relation of such work to the not yet achieved preparation of photosynthetically active extracts. The growth of knowledge of the process of fermentation after Buchner prepared active cell free extracts from yeast suggests that only when similar extracts with photosynthetic activity are available will the chemical study of the mechanism of the photosynthetic reaction and isolation of the participating enzymes be possible. Although the extracts at present obtainable do not reduce CO_2 nor evolve O_2, they do act as photosensitizers for the oxidation of ascorbic acid as is described below.

Stoll has named the chlorophyll-protein compound "chloroplastin." However, as bacteria and blue-green algae do not seem to have chloroplasts, the pigment in these organisms occurring in solution in the protoplasm, we prefer the more general name "photosynthin." Smith, following Mestre's terminology, calls the extract from spinach "phyllochlorin," but this term usually refers to a specific derivative of chlorophyll.

After various attempts at obtaining the pigment complex of purple bacteria in the form of protein compounds by grinding (French, 1940), and lysis by freezing we gave up these methods and turned to the well known supersonic vibration method for breaking the cells (Chambers and Flosdorf). We have prepared bacterial photosynthin in crude solution by supersonic vibration of suspensions of bacteria in water and have studied the absorption spectra and properties of the extracts from several species but have not yet been able to isolate the pure substances. The preparation and properties of photosynthin, and the photosensitization of ascorbic acid oxidation by it, are discussed here; the absorption spectra are presented in the following paper.

EXPERIMENTAL

The Bacteria Cultures

Pure cultures of *Streptococcus*, (*Rhodopseudomonas* or *Phaeomonas*), *varians* (strains "original" and "C 11"); *Spirillum*, (*Rhodospirillum*), *rubrum* (strain "S 1"); *Rhodovibrio sp.* (strain Gaffron); and *Phaeomonas sp.* (strain Delft) were kindly given us by Professor C. B. van Niel of the Hopkins Marine Station, Pacific Grove, California. They were kept as stab cultures, then grown for use in the following medium made up in tap water.

NH_4Cl	0.1 per cent
KH_2PO_4	0.05 per cent
$MgCl_2$	0.02 per cent
$NaHCO_3$	0.084 per cent
Na malate	0.075 per cent
Yeast extract (Difco)	0.5 per cent

Both *Chlorella* culture flasks (Warburg, 1919) and sterile bottles protected with an inverted beaker (Eymers and Wassink, 1938) were used for incubation at 35° in the light of several incandescent lamps. The bacteria were centrifuged out of the medium and suspended in tap water; they were then exposed to the high frequency vibration.

The Vibration Apparatus

The earlier experiments were done with an oscillator after designs of Professor G. W. Pierce constructed by Dr. G. R. Tatum which delivers 1.3 amps. driving current at a frequency of 15,000 cycles per second using a water-cooled cup to hold the liquid. Later a more powerful oscillator was constructed for Professor Pierce by Dr. Paul King which was kindly placed at our disposal. This was used at a frequency of 21,000 cycles per second and had a larger cup. The usual output of the oscillator into the activating coil of the magnetostrictive vibrator was 5 amps. A current of 0.4 amps. D.C. passed through the magnetizing coil. During the periods of exposure a stream of water at about 15°C. was forced into the nickel vibrator tube and came in contact with the under part of the 0.025 inch monel disc which formed the top of the tube and bottom of the glass cup which was attached with a thin-walled rubber tube and held the bacteria suspensions, which were thus kept cool. The usual time of irradiation of a 10 cc. batch was 20 minutes which sufficed for practically complete lysis.

The Effects of Sonic Vibration on the Bacteria

When a suspension of bacteria is exposed to vibration in the cup it gradually becomes more transparent and the spotty appearance caused by swirling a suspension of uniformly shaped bacteria disappears. If then the mixture is centrifuged there is a very thin layer of black substance on the very bottom of the tube, above this some white cellular debris collects and the liquid above becomes clear, or in some cases slightly cloudy. It is deep red or brown depending on the species of bacteria. On standing, such solutions gradually become opalescent and eventually develop a precipitate

probably consisting of denatured protein. The observed results of the vibration are believed to be due merely to the rupture of the cell wall.

The effect of the vibration for various periods of time on a suspension of *Streptococcus varians* (C11) is shown in Fig. 1 where the amount of intact cells as judged by the pigment liberated, measured spectrophotometrically, is compared with the remaining capacity to reduce carbon dioxide with gaseous hydrogen. The latter was measured in Warburg manometers in 0.05 M $KHCO_3$ with 5 per cent CO_2 in H_2 in the gas space (French, 1937).

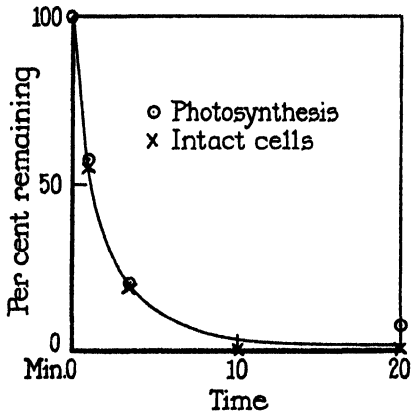

FIG. 1

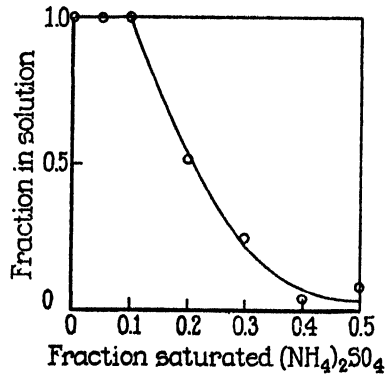

FIG. 2

FIG. 1. A suspension of *Streptococcus varians* (C 11) was treated with supersonic vibration and samples taken at various times. The circles show the relative rate of photosynthesis of the different sámples and the crosses give the fraction of the still intact cells.

FIG. 2. The relative solubility of photosynthin from *Spirillum rubrum* in neutral solutions of $(NH_4)_2SO_4$ of different concentrations.

Apparently the photosynthetic ability decreases in proportion to the cell breakdown under these conditions. It is to be hoped that further experiments will show conditions under which the photosynthetic activity is retained in spite of the cell destruction. The extracts do have strong catalase activity, showing that at least this enzyme is not destroyed by the vibration. An attempt was made to use the H_2 activating enzyme of *B. coli* (Woods, 1936) in conjunction with the pigment of the purple bacteria to reproduce the natural cells' photosynthetic system. Woods has found that *B. coli* grown on formate broth will catalyze the reaction $H_2 + CO_2 \rightleftarrows HCOOH$ in either direction to the point of thermodynamic equilibrium. We have confirmed the uptake of H_2 and CO_2 by *B. coli*. However, in the attempt to obtain the enzymes in solution, it was found that under our

conditions of vibration an exposure of the cells for 2 minutes completely destroys the capacity of a culture to carry on this process.

Properties of the Extracts

The breaking of the cell wall by supersonic vibration is believed not to cause any very great changes in the composition of the cell juice, so a description of the properties of this extract probably bears directly on the question of the nature of the normal cells' photosynthetic apparatus. Concentrated suspensions give a solution so highly colored as to be nearly black. Cloudy preparations which sometimes occur may be made completely clear by saturating the solution with urea. The extract is stable to alkali; it turns greenish and gives a precipitate on treatment with acids. In concentrated HCl it gives a clear green solution with no sharp absorption bands in the visible region. Heating several minutes produces only slight precipitation and a color change to dirty green which fades on boiling. If the solution is shaken with petroleum ether, ethyl ether, or CS_2 the organic solvent layer remains colorless. The addition of considerable quantities of methyl alcohol or acetone to the solution, followed by extraction with any of the above solvents, removes the pigments to the organic solvent and gives a precipitate of denatured protein.

The juice as prepared is of course a mixture of inorganic salts, organic substances, enzymes, and various proteins as well as photosynthin. The colored photosynthin is completely precipitated by neutral $(NH_4)_2SO_4$ at half saturation and will partially redissolve again in water. If more of the salt is added to the supernatant liquid to produce full saturation a small amount of a colorless protein is obtained. Half saturation with urea will prevent precipitation of the photosynthin by half saturated $(NH_4)_2SO_4$.

The solution of photosynthin gives a negative test for tyrosine with Millon's reagent. Both copper and lead salts produce a colored precipitate from which the original colored solution cannot be regenerated by treatment with H_2S.

Fullers' earth acts as an adsorbent for the photosynthin from solutions below pH 9, and gives up the coloring matter in more alkaline solutions. In a Tswett adsorption column fullers' earth colors an even brown the whole length of a 5 cm. column when the water extract is poured through it. If the solution is diluted with phosphate buffer of pH 6.0 all the color is adsorbed in the first 1.5 cm. After adsorption all the pigment can be washed through the column and recovered in solution by concentrated NH_4OH. With $CaCO_3$ in the column there was very little adsorption while with MgO the adsorption was complete in the first 3 mm. So far

it has not yet been possible to get bands of different colors in a Tswett column.

The color of the extract corresponds to that of the cell suspension. Thus, aerobically grown cultures of *Streptococcus varians* are red, as are photosynthin solutions obtained therefrom, while extracts from cultures developed under anaerobic conditions are brown in agreement with the color of the cells (French, 1940). Attempts to change the color of the extract of either the brown anaerobic or red aerobically grown *Streptococcus varians* (orig.) by reduction with colloidal Pd and H_2 or with Na hydrosulfite were not successful. Treatment with dilute H_2O_2 at room temperature gave no color change either, but O_2 was evolved showing the presence of an enzyme with catalase activity.[1] Warming in a 10 per cent solution of H_2O_2 produces complete bleaching.

At the suggestion of Professor E. J. Cohn some of the extract of *Streptococcus varians* (orig.) was dialyzed a long time in cellophane sausage skins against acetate buffers of pH 4.0, 4.5, 5.0, and 5.5 each in concentrations of 2.0, 0.2, and 0.02 M. All the pH 4.0 solutions gave a grayish brown precipitate, leaving no color in solution. At pH 4.5 the precipitate was less altered in color but again left no pigment in the solution. At pH 5.0 there was still no dissolved pigment but the color of the precipitate approached more completely the shade of the original solution. At pH 5.5 some pigment remained in the 2.0 M solution and nearly all stayed dissolved in the 0.02 M buffer. All the precipitates were amorphous on microscopical examination.

To see if the photosynthin could be separated from other proteins by $(NH_4)_2SO_4$ fractionation, an experiment was made with the extract of *Spirillum rubrum*. The protein content of 1 cc. was found to be 41.3 (duplicate 37.2) mg. by precipitation with 10 per cent trichloracetic acid and collection of the precipitate on a sintered glass Gooch crucible. The absorption of this solution was measured at 875 mμ and the coefficient found to be 2.64 cc./mg./cm. 5 cc. were precipitated with 0.5 saturated $(NH_4)_2SO_4$ and the precipitate redissolved. The absorption and protein content of this solution were measured, and the precipitation repeated with 0.3, 0.2, and 0.1 saturated $(NH_4)_2SO_4$, giving the results summarized in Table I.

Since there is no apparent separation into fractions of either greater or less absorption per milligram of protein we may conclude that the various proteins present are not easily separable by salt precipitation at least at neutrality. It may of course be that the photosynthin constitutes the

[1] These experiments were done with Professor A. B. Hastings.

major part of the total protein present in which case little or no separation would be evident by this procedure.

The solution having an extinction coefficient of 2.64 cc./mg./cm. at 875 mμ is equivalent to 19.8 mg./l bacteriophaeophtin; *i.e.*, about 2×10^{-5} M. It contains 41.3 mg. protein per cc. Assuming arbitrarily a molecular weight of 70,000 it would be about 5.9×10^{-4} M in respect to protein. Thus in this particular solution the molecular ratio of green pigment to protein on the basis of the above assumption would be of the order of 1 to 30.

The relative solubility of the colored photosynthin in $(NH_4)_2SO_4$ solutions of varying concentration was determined by making solutions containing different amounts of salt but the same amount of extract of *Spirillum rubrum*. The solutions were centrifuged to remove the insoluble material and the supernatant liquids compared colorimetrically with the solution containing no $(NH_4)_2SO_4$. Fig. 2 shows the results of this experiment. It must be realized that the presence of other proteins which might carry down photosynthin by adsorption may distort both this and the following curve for the effect of pH on the solubility. However, the fractionation experiment above suggests that the major part of the protein present is the one which functions as a carrier of the pigments. This makes the possibility of adsorption a less probable objection to considering these curves as describing the properties of the chemically pure photosynthin itself.

TABLE I

Attempted Fractionation of Photosynthin

Absorption coefficient at 875 mμ per mg. protein in water solution after successive precipitations with $(NH_4)_2SO_4$ solutions.

$(NH_4)_2SO_4$ concentration used for precipitation	Absorption coefficient of redissolved photosynthin
per cent saturation	$\frac{cc.}{mg. \times cm.}$
0	2.64
50	2.74
30	2.84
20	2.52
10	2.76

Buffers of glycine-HCl, acetate, and phosphate were used for the measurement of solubility of *Spirillum rubrum* photosynthin which was determined colorimetrically at various pH values. The results are presented in Fig. 3.

Photoxidation by the Cell Extracts

It has already been stated that the photosynthin solutions obtained from various cultures of purple bacteria do not display the normal photosynthetic activity of the intact cells. An experiment was tried with the extracted purple bacteria juice in which was suspended live *B. coli* in

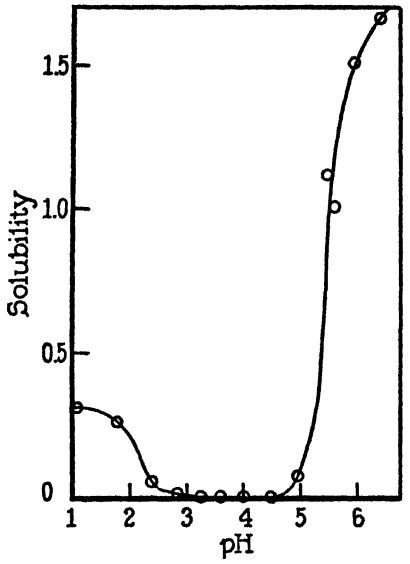

FIG. 3

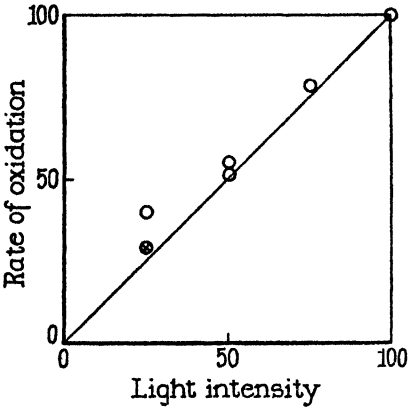

FIG. 4

FIG. 3. The relative solubility of *Spirillum rubrum* photosynthin in M/15 phosphate buffers at various pH values.

FIG. 4. The rate of the photochemical oxidation of ascorbic acid by *Rhodovibrio sp.* photosynthin is proportional to the light intensity. The rate of reaction in the brightest light decreased from 26 to 15 c.mm./10 min. during the course of the experiment (4 hours), so each point has been multiplied by a factor to correct for this change. The point marked with a cross was measured with O_2 in the gas space. V_G = 11.31 cc., V_L = 7.0 cc., pH 6.0, 1 cc. pigment, temperature = 24.4°C.

the hope of using the *coli* enzymes to activate the molecular H_2, while the pigment caused CO_2 reduction by the activated H_2. No difference in rate of $H_2 + CO_2$ uptake was, however, observed in the light and in the dark. These observations thus agree with the negative results of attempts to obtain photosynthetically active extracts from green plants.

The bacterial extracts also fail to show the presence of enzymes activating the oxy-hydrogen reaction. The above mentioned destruction of the enzyme of *B. coli* by exposure to supersonic waves would fit in with the supposition that the hydrogen activating system is relatively unstable.

Ethyl chlorophyllid in acetone solution has been found by Gaffron to be a photosensitizer for the oxidation of an easily oxidizable substance, allyl thiourea, by dissolved oxygen. The quantum yield of this reaction he found to be 1.0 over the range 655 436 mμ. It is possible that this property of chlorophyll of photosensitizing an otherwise fairly stable system may be related to the participation of that substance in the photosynthetic reduction of CO_2.

An analogous situation was found with water solutions of the cell free bacteria juice. Ascorbic acid was used as the O_2 acceptor for the experiment shown in Table II. It is evident that the ascorbic acid will not act as a reducing substance for CO_2 reduction by the illuminated pigment but is susceptible to sensitized photoxidation by air. That the presence of

TABLE II

Photoxidation of Ascorbic Acid

Rectangular vessel V = 18.37 cc.		
Light:	40 watt showcase bulb at 0.5 cm.	
Liquid:	0.5 cc. *Streptococcus varians* (orig.) extract (5.56 per cent solids)	
	0.7 cc. 0.5 M $KHCO_3$	
	1.8 cc. water	
	20.0 mg. ascorbic acid	
Temperature:	23.4°C.	
Gas:	5 per cent CO_2/N_2	5 per cent CO_2/air
	mm./5 min.	*mm./5 min.*
Dark	−1	−2
Light	0	−12

CO_2 is unnecessary is shown by Table III. The spontaneous oxidation of ascorbic acid is sensitive to Cu catalysis (Stotz, Harrer, and King). Light does not, however, influence the rate of this Cu-catalyzed oxidation according to the results of a specifically designed control experiment. Similar photosensitizations of ascorbic acid oxidation have been reported by Martini for lactoflavine and methylene blue (*cf.* Hopkins and also Hand, Guthrie, and Sharp). Puckowitz has recently found that extracts of spinach will act in much the same way as do our solutions also with an apparently low quantum yield.

Measurements were made of the rate of the photochemical reaction in different spectral regions with light from a 500 watt incandescent bulb set up with a silvered watch glass reflector and an f:1 condenser lens throwing the beam on a concave microscope mirror placed in the thermostat water. This threw an oblong image of the condenser lens on the bottom of the rectangular vessel. Three times the photochemical effect was produced

TABLE III

Photoxidation of Ascorbic Acid

Conical vessel

Main space: 2.00 cc. phosphate buffer pH 7.0 M/15

0.5 cc. *Rhodovibrio* extract $\log_{10} \frac{I_0}{I} \Big|_{855\ m\mu}^{1\ cm.} = 560$

Inset: 0.2 cc. 5 per cent KOH

Side arm: 17.0 mg. ascorbic acid

Light: 500 watt projection bulb with f:1 condenser and reflector

Gas: Air

Temperature: 24.7°C.

Time	Illumination	O_2 absorbed
min.		*c.mm.*
10	Light	0
10	Dark	0
Ascorbic acid added from side arm		
20	Dark	0
40	Light	34
60	Dark	7

TABLE IV

Photosensitization of Ascorbic Acid Oxidation by Bacterial Extract in Different Spectral Regions

Rectangular Vessel V = 18.09 cc.

Liquid: 6.00 cc. phosphate buffer M/15 pH 7.0 (pH with ascorbic acid = 4.4)

1.00 cc. *Rhodovibrio* extract $\log_{10} \frac{I_0}{I} \Big|_{855\ m\mu}^{1\ cm.} = 560$

50 mg. ascorbic acid

Light: 500 watt projection bulb with f:1 condenser and reflector

Light path in water = 27 cm.

Gas: Air

Temperature: 24.4°C.

Wave length region	Filter	Rate of O_2 uptake
mμ		*c.mm./10 min.*
920–400	None	−34
920–600	Wratten No. 29	−13
920–650	Wratten No. 70	−13
920–800	Wratten No. 70, No. 74	−4
800–400	3.2 cm. saturated $FeSO_4$ in 10 per cent H_2SO_4	−13
650–400	3.2 cm. 5 per cent $CuSO_4$	−10
Dark		−1.1

by this arrangement as by a long 40 watt frosted show case lamp about 0.5 cm. below the vessel. Filters were placed in the beam as desired. The results of this experiment are summarized in Table IV. At the position of minimum absorption, 650 mμ, the absorption of the solution as used in the vessel was 68 per cent; at all other wave lengths the solution absorbed more strongly. This experiment shows that both the near infrared region and the visible region are capable of action, thus ruling out the possibility of the effect being solely due to some other pigment than the infrared absorbing photosynthin.

Another experiment with this illumination arrangement and neutral filters made by diluting India ink with water and calibrating the solutions spectrophotometrically showed approximate proportionality between light intensity and rate of reaction corrected for both dark reaction and decrease of the rate in bright light with time. Up to the limit of light intensity obtainable with this apparatus there is direct proportionality between the intensity and the rate of photochemical action as shown in Fig. 4. With O_2 instead of air the rate of the oxidation in the dark was raised from 2.5 to 10.5 c.mm. O_2 per 10 minutes but the rate of photoxidation corrected for the thermal reaction rose only from 15 in air to 25 c.mm. O_2 per 10 minutes in O_2.

DISCUSSION

The results here reported are of significance in that they contribute to the knowledge of the behavior of one constituent of the cells' photosynthetic system. Here we are dealing presumably with the pigment under the same conditions as it exists in the live cells. However, the other constituents of the system, enzymes, and possibly intermediate products, are evidently destroyed or diluted to such an extent that their activity is no longer measurable after this extraction process. There is of course the possibility that destruction of the cell structure or disturbances in the relation of various cell parts to each other destroy the photosynthetic capacity, although the substances taking part in the reaction are unaltered and still present in solution.

In the determination of quantum yields of photosynthesis it is necessary to measure the fraction of the incident light absorbed by the pigment in the cells. The cell wall scatters a good deal of light (French, 1937) especially in the visible spectrum, thus complicating measurement of the truly absorbed portion which controls the metabolic activity. The quantitative extraction of the pigment from the cells by the supersonic treatment described here gives better means of determining the light absorbed by the

pigment alone in a suspension of bacteria, since the absorption of the pigment may be measured in a clear solution. It would also be extremely important to study the extracts of blue-green algae which have the photosynthin dissolved in the protoplasm rather than in the solid structure of chloroplasts.

The starting point for this work was the desire to isolate the photosynthin in chemically pure form and to determine its composition as to pigment, protein, metals, and other possible constituents such as ether soluble fractions and carbohydrates. The completion of this program would involve work on a larger scale than has yet been attempted. A determination of the molecular weight by the ultracentrifuge would be interesting and centrifugal studies are necessary to confirm the present idea that the two types of pigment, bacteriochlorophyll and carotinoids, are attached to the same protein molecule. This conception is based on the facts that fractionation of the extract with $(NH_4)_2SO_4$ solution or by acid precipitation does not produce fractions of different colors, and that such fractionation has also been impossible either by adsorption on a Tswett column or by spreading on filter paper. A few preliminary experiments with the addition of acetone or methyl alcohol to the cold solution of photosynthin under ether with the hope of removing either of the pigments from the protein and then making it recombine by evaporation of the ether were not successful but should be continued.

SUMMARY

1. Photosynthetic bacteria in water suspension break open when treated with supersonic vibration thus liberating the cell contents, including a water soluble protein to which is attached the otherwise water insoluble pigments, bacteriochlorophyll and carotinoids. Both types of pigments appear to be combined with the same protein.

2. The protein pigment compound is insoluble in the region of pH 3.0 to 4.5 and in neutral solution can be completely precipitated by 0.5 saturated $(NH_4)_2SO_4$. It is soluble in distilled water and adsorbable on fullers' earth.

3. Supersonic extracts of photosynthetic bacteria do not have the ability to carry on photosynthesis, but will act as a photocatalyst for the oxidation of ascorbic acid with visible or infrared radiation. The rate of the photochemical oxidation is proportional to the light intensity.

It is a pleasure to thank Professor C. B. van Niel for the pure cultures of the bacteria and his help with the manuscript; Professor G. W. Pierce for

the use of the vibration equipment and Drs. G. R. Tatum, Atherton Noyes, and Paul King for their kind assistance; Professors Otto Warburg, A. B. Hastings, and James Franck for many valuable suggestions.

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THE PIGMENT-PROTEIN COMPOUND IN PHOTOSYNTHETIC BACTERIA

II. The Absorption Curves of Photosynthin from Several Species of Bacteria

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INTRODUCTION

In a previous paper (French, 1940*a*) the culture of the photosynthetic purple bacteria and the treatment of the bacteria with supersonic vibration to obtain solutions of their water soluble protein-pigment compounds was described. Spectral absorption curves have been made in the visible and near infrared of photosynthin solutions obtained from four different species of bacteria.

Extraction of the pigments by organic solvents changes the position of their absorption bands in the spectrum (van Niel and Smith, 1935; French, 1937) but extraction of the photosynthin from the cells without destruction of the pigment-protein complex leaves the absorption bands in the same position and with the same absolute height. This indicates that in these extracts the substance with which we are dealing is the same or at least not greatly different from the photocatalyst as it acts in the process of photosynthesis in the living material. From the examination of the visible and infrared absorption spectra of four species of bacteria we find evidence for the existence of three different types of photosynthin.

EXPERIMENTAL

Spectrophotometer Construction

A Hilger 90° constant deviation spectroscope with a dense flint glass prism, which had a drum wave length scale reading to 800 mμ was made into a monochromator by fitting an exit slit from another similar instrument in place of the eyepiece. In order to make it usable farther out toward the infrared the prism was shifted in relation to the wave length scale which was then recalibrated in the visible region with thirty lines from mercury, argon, helium, copper, krypton, and sodium spectra. In the region from 800 to 1,000 mμ the calibration was done photographically as follows. A mercury arc was set up in front of the entrance slit and the green 546.1 mμ line set exactly on

the entrance slit by adjusting the wave length drum. The collimator tube and exit slit assembly was removed and replaced with a camera. Without disturbing the drum setting the camera was adjusted to focus the green line on the plate.

An exposure was made of the mercury spectrum on an Eastman IQ infrared plate sensitized with NH_4OH. Without moving the plate the drum was rotated to bring the near infrared region on to the approximate spot occupied by the green line and an exposure of a cesium spectrum made overlapping the mercury spectrum. Cesium bromide in the hollow of a carbon arc was used as a source. The scale reading was recorded and shifted to another position and the exposure of the cesium spectrum repeated on a fresh part of the plate. The drum was turned back to the spot which placed the green mercury line in its previous position and that spectrum was superimposed on the second cesium exposure. This process was repeated giving five different positions of the cesium spectrum superimposed on one position of the mercury spectrum, the green line of which indicated the exact place taken by the slit. The plate was developed and the distance from the green line to the cesium lines 852.1, 894.4, and 917.2 mμ measured with a micrometer microscope. Large plots were made of the wave length scale reading against micrometer scale reading for each line and the value which would put the line in question on the green mercury line and hence on the slit was determined by interpolation.

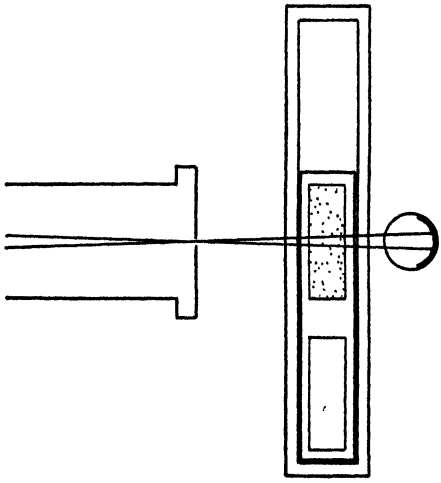

FIG. 1. Photocell arrangement

Another plate was made similarly using the 793.3 line of copper as a reference line and argon 811.5, 912.4, and 965.9 mμ for the calibration. The mercury 1014.0 mμ line was also used. After this procedure the collimator tube with the slit was replaced and several visible lines checked. The photographic calibrations from 811.5 to 1014.0 mμ and the visible lines 809.3 to 404.7 mμ gave a continuous smooth curve from which the scale reading for every 10 mμ was tabulated.

The image of a six volt 1,000 lumen single straight coil filament lamp was focused on the entrance slit of the monochromator with a 10 cm. focal length lens. After emerging from the second slit the light passed through the parallel sided glass absorption vessel of 1 cm. thickness containing the solution to be measured. It was arranged so that a similar vessel containing the solvent alone could be interchanged by means of a sliding rack. Directly behind the absorption vessel was a vacuum cesium photocell, R. C. A. No. 922 arranged as shown in Fig. 1.

The current from this cell was amplified with a single tube circuit using a 6J5 tube and read on a Leeds and Northrup wall galvanometer of approximate sensitivity of 4,000 mm. per μa. The dark current was balanced out with a battery and variable

resistances. For the approximate adjustment of the latter a milliammeter was thrown into the circuit in place of the much more sensitive galvanometer. The collimator tube projected into a light-tight grounded metal box containing the absorption vessel rack, photocell, and amplifier. The filament battery was enclosed in a separate metal box connected with a shielded cable. The galvanometer leads were shielded and the monochromator grounded. In order to reduce the drift, which was nevertheless troublesome, the tube was heated continuously day and night except when charging the battery. Approximate widths of the spectral region (taken as twice the slit width) isolated by the monochromator necessary to give 100 mm. galvanometer deflection at different wave lengths and the dispersion of the instrument are shown in Table I. When working in the infrared, Wratten filter No. 70 or No. 29 was used to remove visible scattered light. Still more important, due to the infrared sensitivity of the photocell, was a filter of 2 cm. of 5 per cent $CuSO_4$ in water for absorption of stray infrared when working in the visible spectrum.

TABLE I

Spectrophotometer Characteristics

Wave length	Width of band isolated by 0.1 mm. slit	Approx. band width for 100 mm. galvanometer deflection
$m\mu$	$m\mu$	$m\mu$
450	0.55	5.5
500	0.80	4.0
600	1.4	2.5
700	2.1	2.7
800	3.1	2.8
900	4.7	4.2
950	5.8	8.6

The linearity of galvanometer deflection with light intensity at the photocell was checked by measuring the extinction coefficient of a piece of green glass with a Koenig-Martens visual spectrophotometer at 580 and 560 mμ and at the same wave lengths with the photoelectric spectrophotometer. Weakening a beam from 100 to 30.9 or to 16.9 on the basis of visual measurements gave respectively 28.3 and 17.5 with the photocell system which was considered to be a satisfactory check.

If I_0 be the galvanometer deflection for a given wave length with the control vessel of solvent in the light path and I the deflection with the vessel containing the solution, then the extinction, E, is given by: $E = \log_{10} \frac{I_0}{I} = K\,cd$ where K is the specific absorption coefficient at wave length λ, c is the concentration of the absorbing substance, and d is the thickness of the cell (1 cm.).

Since the photosynthin has not been isolated as a pure substance we cannot determine absolute values for K.

Procedure and Results

Several extracts were made at different times from *Spirillum rubrum* (strain S1) and the absorption curves of these solutions are presented in

Fig. 2. Both saturated urea and dilute NH_4OH were a great help in clarifying opalescent solutions but did not influence the position or height of the absorption bands. Occasionally a perfectly clear extract was obtained directly in water but the absorption curves were made in filtered saturated urea solution to avoid light scattering in the shorter wave lengths.

In order to determine the relative numbers of molecules of the green bacteriochlorophyll and the red spirilloxanthin in the cell juice the two pigments were separated by organic solvents. Using a sample of pure bacteriophaeophytin kindly supplied by Professor C. B. van Niel as a

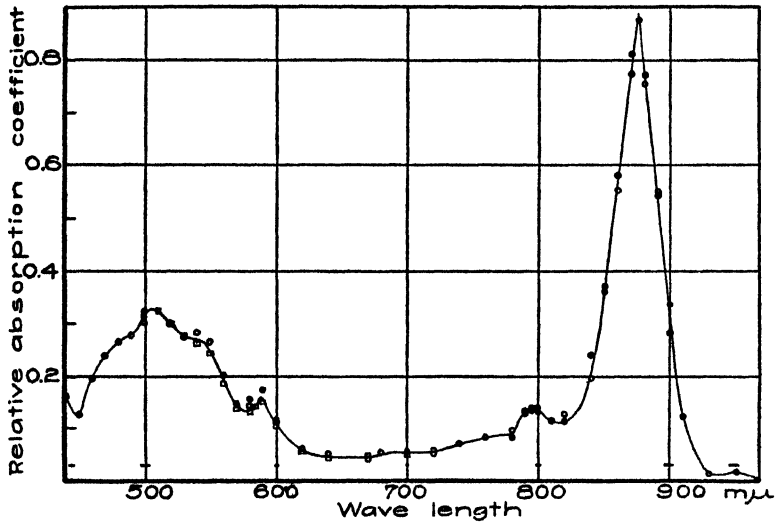

FIG. 2. Absorption curve of *Spirillum rubrum* extract. Open circles: diluted with NH_4OH; solid circles: diluted with saturated urea solution; squares: diluted with water, visual determinations. The curves are based upon data obtained with different extracts and have been brought together by using the appropriate factors. The slit width is shown by bars at the bottom of the figure.

standard, the amount of this pigment present was determined by absorption measurements in chloroform. The absorption curve of this substance was measured and is shown in Fig. 3. The absorption of the spirilloxanthin was measured in CS_2 and the concentration of pigment present taken from the data of van Niel and Smith.

The procedure used in the pigment concentration measurements having unfortunately been carried out before the publication of van Niel and Arnold's method was as follows:

Bacteriochlorophyll Determination.—In the dark 2.55 cc. of cold *Spirillum rubrum* extract prepared from a suspension containing 30 c.mm. cells/cc. were added to 22.5 cc.

of cold methyl alcohol. In this 90 per cent methyl alcohol the red pigment and the protein is insoluble while the green pigment dissolves. The precipitated protein was centrifuged down and washed in the dark with cold 90 per cent methyl alcohol. The

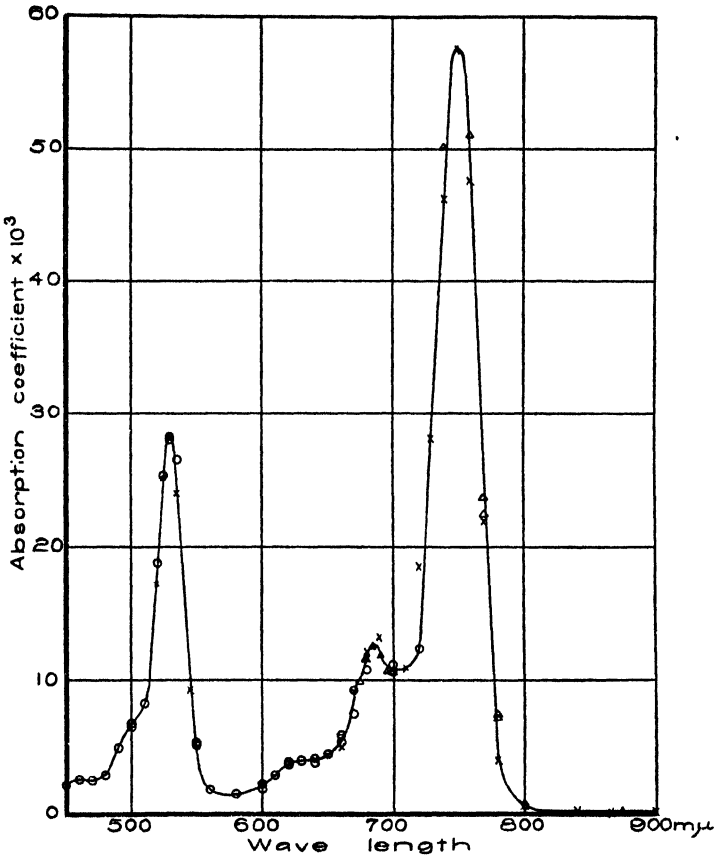

FIG. 3. Absorption curve of bacteriophaeophytin kindly given us by C. B. van Niel. It was prepared from *Spirillum rubrum*. Visual determinations are shown by (o) and photoelectric ones by (x) and (Δ). Two samples gave 28.3 and 28.0 × 10^{-3} for K at 530 mμ in the equation:

$$\log_{10} \frac{I_0}{I} = Kcd \text{ where } c \text{ is in mg./l and } d \text{ in cm.}$$

methyl alcohol solution and washings were combined and poured into cold ether. Four washings in the dark with cold water removed the methyl alcohol from the ether solution of bacteriochlorophyll which was then shaken with 20 cc. of 10 per cent HCl to turn the bacteriochlorophyll into bacteriophaeophytin which is rather stable to light. The water layer remained colorless. The ether solution was washed with water to remove

HCl, allowed to stand over two batches of $CaCl_2$ to remove water, then evaporated on the steam bath. The residue of bacteriophaeophytin was taken up in 4.1 cc. of chloroform. This solution showed a yellower tinge than a solution of pure bacteriochlorophyll probably due to a trace of spirilloxanthin dissolving in the 90 per cent methyl alcohol. At 530 mμ the extinction, $\log_{10}\frac{I_0}{I}$ of 1 cm. of this solution was 0.340.

From Fig. 3 the absorption coefficient of a solution of pure bacteriochlorophyll from this same strain of bacteria is 28.2×10^{-3} at 530 mμ, that is:

$$\log_{10}\frac{I_0}{I} = 28.2 \times 10^{-3}\, cd$$

with c in mg./l and d in cm. Using this constant for the determination of the concentration of the pigment in solution:

$$C = \frac{E}{Kd} = \frac{0.340}{28.2 \times 10^{-3} \times 1} = 12.05 \text{ mg./l}$$

the original solution then contained:

$$\frac{4.1}{2.5} \times 12.05 = 19.8 \text{ mg./l}$$

Assuming a molecular weight of 990 for bacteriophaeophytin,

$$\frac{19.8}{990{,}000} \cdot 2.00 \times 10^{-5} \text{ M}$$

Spirilloxanthin Determination.—2 cc. of the bacterial extract were shaken with CS_2. The CS_2 remained colorless. Acetone was added to denature the protein and liberate the pigment, then the mixture was shaken. More water was added and the CS_2 which separated after centrifuging contained all the red pigment. The CS_2 was made up to a volume of 20.8 cc. and the absorption at 530 mμ measured with a Koenig-Martens spectrophotometer. For a 1 cm. layer $\log_{10}\frac{I_0}{I} = 0.35$. Van Niel and Smith give 14.8×10^4 as the molecular absorption coefficient at 530 mμ in CS_2 of pure spirilloxanthin prepared from this strain of bacteria. Using this value to determine the concentration of our CS_2 solution, $x = \frac{0.35}{14.8 \times 10^4 \times 1} = 23.6 \times 10^{-6}$ M. For the concentration of the spirilloxanthin in the extract: $C = \frac{20.8}{2.0} \times 2.36 \times 10^{-6} = 24.6 \times 10^{-6} = 2.46 \times 10^{-5}$ M. In our supersonic extract of *Spirillum rubrum* then:

$$\frac{\text{Concentration of bacteriochlorophyll}}{\text{Concentration of spirilloxanthin}} = \frac{2.00 \times 10^{-5}}{2.46 \times 10^{-5}} = 0.81$$

In Fig. 4 is shown the absorption curve of a supersonic extract of *Rhodovibrio sp.* (*Rhodobacillus*) (strain Gaffron) in saturated urea solution. Open circles are points obtained photoelectrically while the closed circles were obtained with the Koenig-Martens instrument on the same solution.

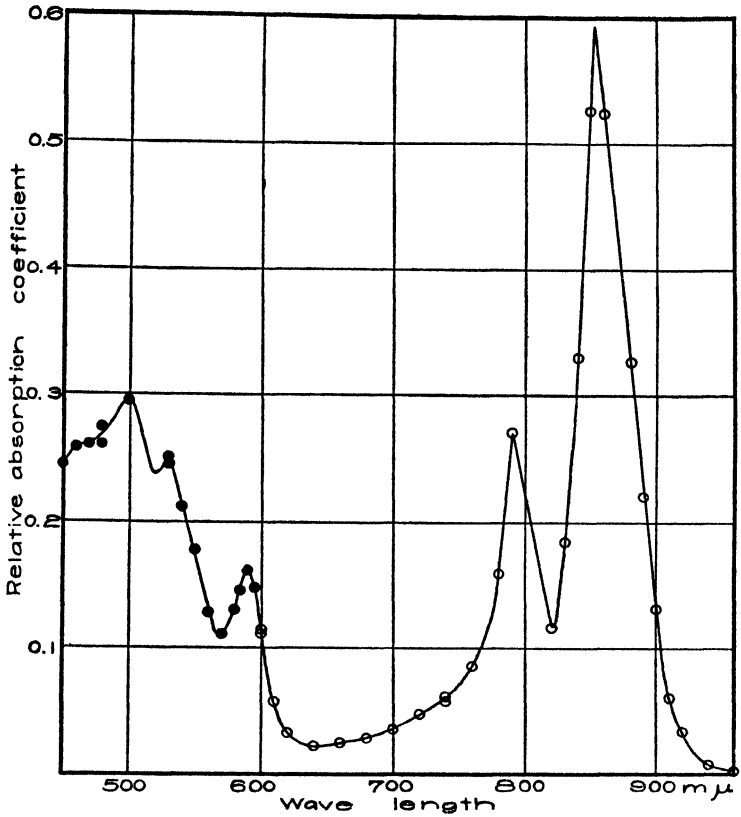

FIG. 4. Absorption curve of *Rhodovibrio sp.* (strain Gaffron) extract in saturated urea solution. Visual measurements are shown by closed circles and photoelectric ones by open circles.

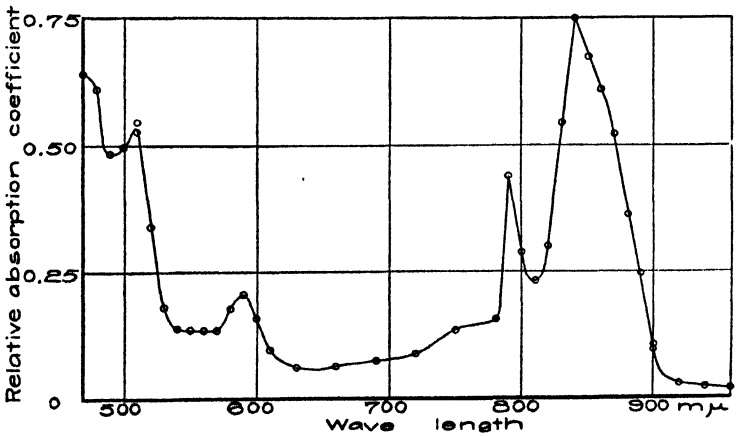

FIG. 5. Absorption curve of *Phaeomonas sp.* (strain Delft) extract in saturated urea solution.

A water supersonic extract was also made of *Phaeomonas* (*Phaeobacillus*) *sp.* (strain Delft) and was similarly diluted with saturated urea for absorption measurements. The curve of Fig. 5 is for this species.

An absorption curve was made of a water extract of aerobically grown brown *Streptococcus varians* (strain C11) diluted with saturated urea and is given in Fig. 6.

With a spectrocolorimeter a supersonic extract was compared with a suspension of living bacteria. In the visible spectrum the absorption

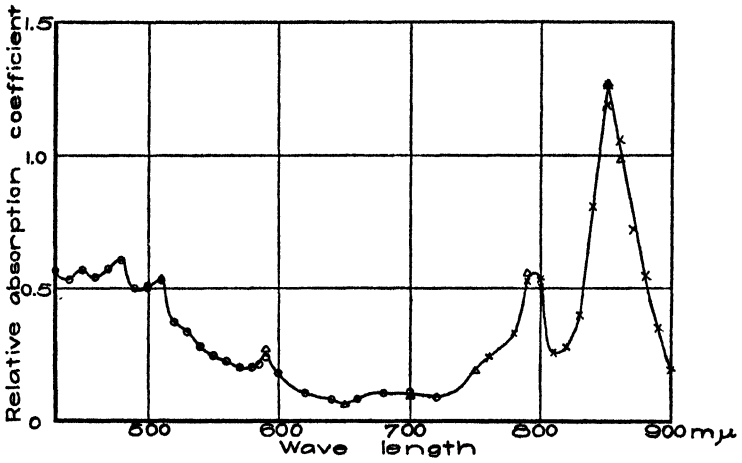

FIG. 6. Absorption curve of cell juice of brown anaerobic *Streptococcus varians* (strain C11). Triangles and crosses are photoelectric measurements with two different solutions. The circles are visual measurements with a third solution. The dots and crosses were brought to the height of the circles by using conversion factors; the concentrations were unknown. At 855 mμ $\log_{10} \frac{I_0}{I} = 6.4 \times 10^{-12}\, cd$ where d is in cm. and c is in number of cells/cc., or $\log_{10} \frac{I_0}{I} = 0.26\, cd$ where c is in c.mm. cells/cc.

bands are in the same position both in the live bacteria and in the extracts. In order to extend this observation to the infrared bands and to find if the bands changed either in position or in height the following experiment was made with *Streptococcus varians* (C11). In each of two test tubes 10 cc. of a bacterial suspension were placed. One was diluted with 10 cc. of water and the other with 5 cc. of 30 per cent H_2O_2. The tube with H_2O_2 was then warmed to 60°C. to bleach the pigments. After a few minutes another 5 cc. portion of the H_2O_2 was added. This treatment gave colorless bacteria to use as a light scattering control for pigment absorption

measurements with the other batch of unbleached cells. The suspensions were diluted and placed in two 1 cm. absorption vessels and illuminated with monochromatic light in the spectrophotometer. As the photocell was directly behind the vessels and of considerably larger area than the cross section of the light beam practically all of the light scattered by these suspensions fell on the photocell as indicated in Fig. 1. I was taken as the galvanometer deflection with the normal bacteria suspension and I_0 as the deflection at the same wave length with the bleached suspension in place. In this way an infrared absorption curve of the pigment *in situ* was obtained approximately corrected for light scattering by the bacteria. Some of the normal suspension was then treated with supersonic vibration liberating the pigment from the cells. The centrifuged cell free pigment solution was diluted with saturated urea and an absorption curve made of this extracted pigment as usual. The two curves of Fig. 7 agree well as to the position of the absorption bands and fairly well as to their relative heights. It is therefore to be concluded that the supersonic extraction does not change the condition of the pigment as much as does extraction with organic solvents which causes large shifts in the position of the bands.

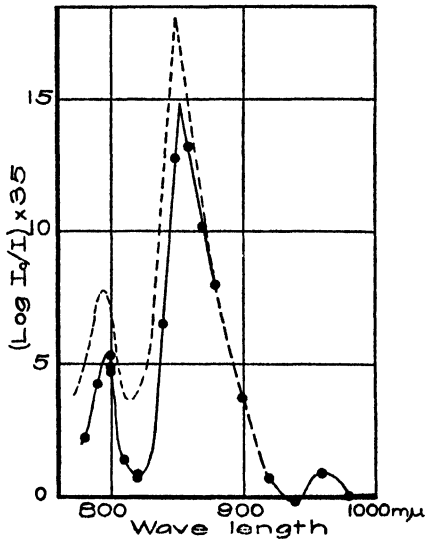

FIG. 7. The absorption curve of the extracted pigment, dotted line, as compared with the absorption of the pigment in live cells of *Streptococcus varians* (strain C11).

On one occasion a curve was made of a *Streptococcus varians* (C11) extract in the infrared region which was identical with the curve of Fig. 6. After standing overnight in the spectrophotometer vessel the measurements were repeated in the morning. The large band at 855 mμ was in the same position though lower by about 10 per cent. Strangely, however, there was no trace of the smaller 795 mμ band. This disappearance of one band without change in the other suggests that the two are due either to different molecules or to widely separated groups of the same molecule. A concentrated solution which had also stood overnight exposed to air retained both bands. Since the addition of small amounts of H_2O_2 or of Na hydrosulfite does not produce

TABLE II

The Height of the Absorption Bands and the Per Cent of Solid Matter in a Supersonic Extract of Streptococcus varians (Orig.)

	Solids	Absorption per mg. at 585 mμ
	per cent	$\frac{cc.}{mg. \times cm.}$
Extract as prepared	5.56	0.340
Extract after dialysis	4.33	0.396
Extract after precipitation, partial redissolving, and dialysis	5.90	0.535

TABLE III

Absorption Bands of Live Cells and Water Extracts of Various Purple Bacteria

Species		Bacteriochlorophyll bands					Carotinoid bands			
		α	β	γ	Δ	ε	α	β	γ	Δ
Spirillum rubrum	Wave length	950	875	795	590		550	510	480	
(Sl) water extract	Relative height	0.022	1.00	0.16	0.18	?	0.32	0.37	0.30	?
**Spirillum rubrum*	Wave length		880	800	590	420	550	515	490	?
(Sl) live cells										
Rhodovibrio sp.	Wave length		855	800	590	?	530	500	460	
(Gaffron) water extract	Relative height		1.00	0.65	0.27	?	0.40	0.46	0.38	?
Phaeomonas sp.	Wave length		840	790	590			510	470	
(Delft) water extract	Relative height		1.00	0.59	0.28	?		0.73	0.85	?
†*Streptococcus varians*	Wave length				590		550	505	475	450
(orig.) (red) water extract	Relative height				0.28	?	0.66	0.92	1.00	0.96
†*Streptococcus varians*	Wave length				590			505	475	450
(orig.) (brown) water extract	Relative height				0.27	?		0.81	1.00	0.43
Streptococcus varians	Wave length		855	795	590			510	480	450
(C11) (brown) water extract	Relative height		1.00	0.42	0.19	?		0.40	0.46	0.43
Streptococcus varians										
(C11) live cells										
Photoelectric	Wave length	950	855	795	590	?		510	480	?
Photographic	Wave length		860	800	(Visual)			(Visual)	(Visual)	

* French, 1937.

† French, 1940*b*.

? Not looked for.

any change in the color or the infrared band heights the disappearance of the 795 band was not due to an easy oxidation or reduction.

As the photosynthin has not yet been isolated it is possible to give the

heights of the absorption bands only in relative units. For one species, *Streptococcus varians* (orig.), the height of the visible band with a maximum at 585 mμ was determined on the basis of dry weight of the extract. 3 liters of yeast extract medium containing 0.05 M $KHCO_3$ gave about 10 cc. of a pasty cell suspension on centrifuging. From this was obtained by supersonic irradiation and centrifuging 7 cc. of clear dark brown liquid. One part of the solution was dried over H_2SO_4 in vacuum and weighed, one part used for absorption measurements, one part dialyzed first then used for an absorption measurement and later dried and weighed. Another part was precipitated with 0.5 saturated $(NH_4)_2SO_4$, centrifuged, and the precipitate extracted with water. The solution so obtained was used for an absorption determination and then dried and weighed. The data are given in Table II. (*Cf.* Table I in the previous paper.[1]) At the maximum of the red pigment absorption band (475 mμ in this solution) the absorption per mg. is 1/7400 that of an equal weight of the pure protein free crystallized spirilloxanthin of van Niel and Smith in CS_2.

DISCUSSION

The position and relative heights of the various absorption bands in different species of bacteria are given in Table III. It is evident that the band at 795 and that at 590 mμ occur in all the species examined. Differences in position of 5 mμ are not considered to be of significance in these determinations. The larger band is, however, found to occur at three different wave lengths, about 875, 855, and 840 mμ in different species. This may either be interpreted as indicating the existence of three different forms of bacteriochlorophyll or three different proteins which differently affect the position of the large absorption band. The work of van Niel would seem to indicate that only one bacteriochlorophyll exists so that the differences in position of these absorption maxima are more probably due to differences in the protein constituent. The chemistry of bacteriochlorophyll has been recently reviewed by Stoll and Wiedermann.

Addendum.—Two articles on the spectra of water extracts and live cells of purple bacteria, green, and blue green algae by Katz and Wassink and by Wassink, Katz, and Dorrestein have just appeared which give more precise spectra in the infrared. In general their conclusions and ours agree well. Of particular interest is their isoelectric point determination of the *Chlorella* photosynthin at pH 3.7 which agrees with our minimum solubility region of *Spirillum rubrum* photosynthin.

Their suggestion that the different infrared bands are due to combination of the

[1] French, C. S., *J. Gen. Physiol.*, 1940, **23**, 475.

bacteriochlorophyll, (which had the same spectrum in alcohol from all species), with various proteins, each producing only one infrared band is consistent with our results. It may lead to the chemical separation of the different proteins using the different infrared band heights as a criterion of the fractionation procedures.

They compare various extracts with the live cells and also find the position of the maxima in close agreement even without a scattering correction, showing that their carborundum powder grinding procedure also makes extracts with essentially unaltered spectral characteristics. Our supersonic treatment would appear to give more complete extraction in a short time than grinding, thus allowing quantitative work, but may well do more harm to enzymes.

SUMMARY

Absorption curves have been obtained in the spectral region of 450 to 900 mμ for the water soluble cell juice of four species of photosynthetic bacteria, *Spirillum rubrum* (strain S1), *Rhodovibrio sp.* (strain Gaffron), *Phaeomonas sp.* (strain Delft), and *Streptococcus varians* (strains C11 and orig.).

These curves all show maxima at 790 and 590 mμ due to bacteriochlorophyll, whose highest band, however, occurs at 875, 855, or 840 mμ depending on the species. The bacteria that appear red rather than brown have a band at 550 mμ due to a carotinoid pigment. An absolute absorption curve of bacteriophaeophytin has maxima at 530 and 750 mμ.

The extraction of cell juice by supersonic vibration does not change the position of the absorption bands or of the light absorbing capacity of the pigment.

It is a pleasure to thank Professors Otto Warburg for many suggestions and C. B. van Niel for the bacteria and valuable criticism. I am also very grateful to Professor G. W. Pierce and his collaborators for the use of the supersonic apparatus, to Professors Theodore Lyman and Otto Oldenburg for the use of the spectroscopic equipment, and to Dr. W. M. Preston for his advice.

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BIOELECTRIC POTENTIALS IN HALICYSTIS

VIII. The Effects of Light*

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Bioelectric effects of illumination are most familiar in animal organs like the retina, but they have also been known in plants since the work of Haake,[1] Klein,[2] Querton,[3] A. D. Waller,[4] and especially J. C. Waller.[5] Other workers[6–11] have also described responses, usually with a rather complex time course, which is difficult to analyze in any consistent fashion. Although some colorless plants (celery,[6] oat coleoptile[10]) give an electric response to light, the phenomena have usually been connected with photosynthesis, as shown by the necessity of chlorophyll, of wave lengths of light absorbed by it, and of CO_2. We have duplicated many of the described effects in this laboratory with various leaves, but it seems doubtful that

* Aided by a grant from The Rockefeller Foundation. It is a pleasure to acknowledge the assistance of Dr. M. L. Darsie, Jr., and Mr. R. K. Skow, under this grant, in many of the experiments here reported.

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[2] Klein, B., *Ber. bot. Ges.*, 1898, **16**, 335.

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[10] Clark, W. G., *Proc. Nat. Acad. Sc.*, 1935, **21**, 681.

[11] Brauner, L., *Protoplasma*, 1937, **28**, 230. Brauner, L., and Brauner, M., *Rev. Fac. Sc. Univ. Istanbul*, 1938, **3**, 1.

such experiments alone can tell us much about the mechanisms relating photosynthesis to the electrical response. First, even in the more simplified aquatic leaves, both the optical and electrical paths are complex; second, virtually nothing is known of the leaf's strictly bioelectric properties in the dark. And the latter information would be difficult or impossible to obtain, since the probable loci of the effects are deep lying mesophyll cells, often shielded from experimental control by impermeable cuticle and non-photosynthetic epidermal cells, and subject to many unknown metabolic alterations.

It is clearly advantageous to study such light effects in large multinucleate cells, such as *Halicystis*, *Nitella*, and *Valonia* (see Marsh[9] for reports on *V. ventricosa*). In these cells, both the optical and electrical paths are much simplified, with the photosynthetically active chloroplasts very close to the electrically active cell surfaces, and the latter accessible to considerable experimental control. Further, in several of these plants there is also a large background of known bioelectric data, which should be useful in interpreting the light effects. With this in view, during the study of other bioelectric effects in *Halicystis*, the author has constantly included light as one of the variables in experimentation. Many of the experiments were performed as long as 6 or 7 years ago, and some of the effects have been briefly alluded to in former papers.[12–14] Only recently, however, has it seemed possible to explain most of the effects on a rational basis, that of an altered ionic permeability and mobility in the cell surface, as developed in the discussion at the end of this paper.

Most of the phenomena can now be referred, primarily, to treatments which alter the P.D. and ionic mobilities in some characteristic fashion, and then, secondarily, to a few simple effects of photosynthesis which influence these treatments in turn, either assisting or counteracting them. Usually this is by oxygen production, or CO_2 consumption with its resultant pH changes. Certain anomalies in the latter were indeed first suggested by the P.D. changes, and corroborated by direct test with the glass electrode.[14] Otherwise, few new assumptions have had to be introduced to account for the effects in *Halicystis*, which may therefore be useful in interpreting the light responses of other single cells, and the more complicated effects in tissues.

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Method

In general the technique is that of previous papers of this series.[12, 13]

Impaled cells of the Californian *H. ovalis* have been mostly employed, although in many instances concordant results have also been obtained with *H. Osterhoutii* in Bermuda. Adequate time for recovery after impalement was allowed, usually overnight or longer. (Illumination tends to hasten recovery soon after impalement; see below.) Measurement was by compensation potentiometry with a sensitive galvanometer as null indicator. Readings were usually taken at 15 second intervals during rapid changes of P.D., or at 1 or 2 minute intervals during slower changes. More recently many records have been taken with the automatic recording potentiometer (Micromax) of Leeds and Northrup, which balances itself every 3 seconds, and faithfully follows moderately rapid potential changes. Both methods can be used with a vacuum tube circuit to avoid even momentary current drain on the cell, but this is not usually necessary. Extremely rapid changes, especially during the first moments of illumination, have in some cases been recorded photographically, using a quick galvanometer or string galvanometer.

The light sources have been daylight (north marine exposure), incandescent lamps, and carbon arcs, all measured with a photronic meter placed as close to the position of the cell as possible. Slightly crumpled aluminum foil reflectors were placed at the backs and sides of the shell vials containing the cells, to equalize illumination around the cell as much as possible. No essential difference has been observed in the effects of the different light sources; in addition, several Corning glass filters of calibrated transmission were used to isolate spectral regions. The effective wave lengths in general corresponded to those of chlorophyll absorption; both red and blue regions of comparable intensity produced good responses, while green was much less effective (enabling many "dark" operations to be carried out in dim green light, to which the eye is very sensitive).

The laboratory was darkened between illumination periods to an average light intensity of less than 1 meter candle; there was no bioelectric response to this dim light. As an added precaution during dark periods, the vial containing the cell was also completely shielded with a cylinder of black paper; this was replaced with the foil reflector during illumination. In exploring the effect of varied light intensity, the same lamp was used throughout, being moved along an optical bench until the desired reading was obtained on the meter. Infrared was largely filtered out by means of a dilute $CuSO_4$ solution in the path of the light; but removal of this filter usually produced no perceptible effect, except in cases where slow heating of the sea water gave rise to complications (*e.g.* with lowered O_2 tension, or with added ammonia). Temperatures were read on a thermometer in the same vial as the cell, and usually not more than 1°C. variations occurred in a given experiment; an extra water bath around the vial was often used to maintain a still more constant temperature. The sea water in the vial itself was also frequently stirred by bubbling gas (air or N_2).

In the present paper, only room temperatures (15 to 22°C.) were employed. Temperature of course influences the photosynthetic rate, and therefore the speed of bioelectric response to light in many cases; its effects will be specifically described in another paper.

Little or no ultraviolet was ordinarily present in the light reaching the cell through

glass barriers; but when a few cells were purposely exposed to ultraviolet from carbon or quartz-mercury arc source, with no glass and a minimum of sea water intervening, no distinctive effect was observed. A few experiments indicated that much longer exposure to intense ultraviolet decreases later response to visible light. This is in agreement with its inhibiting effect upon photosynthesis.[15] My thanks are due to Dr. William Arnold for collaboration in these experiments.

The sign of the P.D. is always understood as that of the outside of the cell as indicated by the external circuit. Thus a *positive* (outwardly directed) P.D. tends to drive current *outward* across the protoplasm, from sap to sea water, and thence to the galvanometer or measuring circuit. Positive P.D. is indicated below the zero line, in the graphs, as throughout this series. Measurements made in the dark are generally indicated by solid circles; those in the light by open circles. (Where Micromax records have been traced, a different convention is followed.) In addition, *L* stands for light, *D* for dark, and arrows indicate the moment of change from one to the other, or other experimental changes.

Effects of Light on the Normal Potential

As pointed out in previous papers,[12, 13] the P.D. of *Halicystis* (when aerated) remains high and unaltered for many days in the dark, probably because in their 10 days to 2 weeks of impaled life the cells do not exhaust all their carbohydrate or other reserves. (*Cf. Valonia ventricosa*[9] which lost most of its P.D. after a much longer dark period.) The P.D. of darkened *H. ovalis* remains close to its original value of 80 mv., or may even rise to nearly 90 mv. shortly before death.

Illumination, even with rather intense light (10,000 meter candles or higher) does not raise this dark value very greatly: at the maximum about 8 mv., or 10 per cent, and usually only 2 or 3 mv. (3 or 4 per cent). The form of this small light response, such as it is, is fairly characteristic, consisting of a quick initial increase of 1 or 2 mv., followed by a fall, after which there may be a slower rise occupying several minutes. A slow decrease may then follow, with continued illumination. On darkening, a converse change occurs, with a small initial decrease, a momentary recovery, and a slower drift toward the steady dark value.

Examples of the normal response are shown in Fig. 1; others are to be seen as controls accompanying experimental modifications in Figs. 8 and 11.

This normal time course in some ways resembles that found in leaves, etc., although it is very much smaller in magnitude. (Changes of 50 to 100 mv. have been found in leaves on illumination.) It much more closely parallels, although in reverse sign, the light effects in impaled *Valonia* (as reported by Marsh[9] in *V. ventricosa*, and verified by the author in that species and in *V. macrophysa*). There again, the actual change in millivolts is usually small

[15] Arnold, W., *J. Gen. Physiol.*, 1933–34, **17**, 135.

under normal conditions, although when expressed as *per cent* of the normally low (negative or inwardly directed) P.D. of *Valonia*, it appears to be more impressive than in *Halicystis*. The normal light effect in *Nitella* is also slight (unpublished observations).

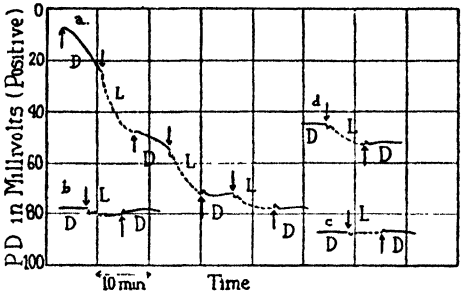

FIG. 1. The effect of light upon the normal P.D. of *Halicystis ovalis*. Curve *b* represents a typical P.D. of about 77 mv. in the dark. When illuminated (5000 meter candles, incandescent source) the P.D. rises about 4 mv., after an initial cusp of an increase and decrease. On darkening there is a reverse cusp and slow fall to almost the original level. In curve *c*, a rather high P.D. of 86 mv. is shown; beyond the initial cusp, light scarcely increases this P.D., nor is there a very great dark effect. On the other hand, curve *d* represents an exceptionally low P.D., reached in a cell which had stood in a very small vessel for some time, partially depleting the oxygen in the sea water. Light here increases the P.D. from 45 to 53 mv., and would have raised it still higher on continued exposure. In the dark the P.D. then remains almost at its preceding light level, not immediately falling. This is due to the oxygen released by photosynthesis.

Finally, curve *a* represents the increased effect of light on the P.D. as it develops in a freshly impaled cell. The first arrow shows the moment of impalement, measurements being begun as soon as possible thereafter. At about 25 mv., the cell was illuminated and the P.D. rose much more rapidly to 48 mv. In the dark it then rose more slowly to 55 mv., in the light rapidly again to 72 mv. In the dark it then was nearly constant, but light drove it to 77 mv., where it remained, in the dark. The greater sensitivity to light during the rise of P.D. is discussed in the text.

Actual tracing of Micromax records, balanced every 3 seconds. Solid lines represent dark observations (*D*), broken lines, light (*L*), arrows indicating time of change.

How then may we reconcile these small effects in single cells with the much larger ones often found in complex tissues? It might of course be suggested that the latter are due to the summation of small effects in a long series of single cells. But this demands either an inherent polarity along each cell (like the radial asymmetry observed in *Halicystis* on impalement), or an appreciable light gradient across each cell. While these may contribute, it seems more reasonable that the large effects are in many cases due to exaggeration of the normal small light effect by various meta-

bolic conditions (decreased O_2, increased CO_2, production of acid or ammonia, etc.) which might prevail in thick, massive tissues. Greatly magnified effects in *Halicystis*, produced by just such agencies, will be described in the next sections.

Before passing on to these controlled effects, it may be well to describe a larger light effect often obtained, which can hardly be classed as "normal," yet might arise under some conditions of experiment. This is the greater sensitivity to light observed soon after the cells are impaled, and before the P.D. has risen to its normal constant value. Light given then hastens the rise of P.D., apparently speeding up recovery from the injury of impalement, since the P.D. does not fall back very much on darkening. The cause of this is not analyzed, but may be connected with the markedly increased respiratory rate which has been measured in this laboratory soon after impalement; this possibly decreases the O_2 tension in or near the cell to a point which lowers the P.D.,[13] and is counteracted by the O_2 released in the cell by photosynthesis (*cf.* next section). Another possibility is that a high internal acidity develops, either as the result of this respiration, or from the mechanical breakdown of barriers during impalement. Such acidity also affects the P.D. (see below); and photosynthesis, by using up CO_2 could counteract it. Possibly some intermediate product of photosynthesis is more readily utilized in healing the wound around the capillary, or in other adjustments, than the more stable reserve carbohydrates. The effect is best avoided by allowing sufficient time for recovery.

A characteristic example is shown in Fig. 1*a*, in comparison with the normal light effects later observed.

A somewhat similar increased sensitivity to light has been observed in freshly impaled *Valonia* cells (unpublished observations).

Another, larger light effect, can also be induced by keeping the cell in the dark very quietly for some time—overnight or longer—especially in a very small vessel. Then the P.D. falls,[13] and is restored markedly by light (Fig. 1*d*). Since this appears to be largely due to lowering of the oxygen content of the small volume of sea water by the cell's own respiration, it is best explained by the next section.

Lowered Oxygen Tension

As indicated in a recent paper,[13] sufficient lowering of the O_2 content of the sea water surrounding an impaled *Halicystis* cell causes the P.D. to fall from about 80 mv. to around 10 or 15 mv., or even less. This may be accomplished by the cell's own respiration in a very small closed vessel (as above) or more rapidly by adding a rapidly respiring organism such as *Ulva* or yeast (*cf.* Fig. 2) but most satisfactorily and clearly, without the complication of CO_2 production and other factors, by bubbling the sea water with a stream of nitrogen containing a low percentage of O_2 (*e.g.*, 0.2 per cent O_2). It was early found that such depression of P.D. occurred only in the dark, or at very low light intensities. Strong illumination promptly restores the P.D., as shown in Fig. 3, with practically the same time course as produced

by re-introduction of air. It seems reasonable to conclude that the effect of light is here due to photosynthetic production of oxygen.

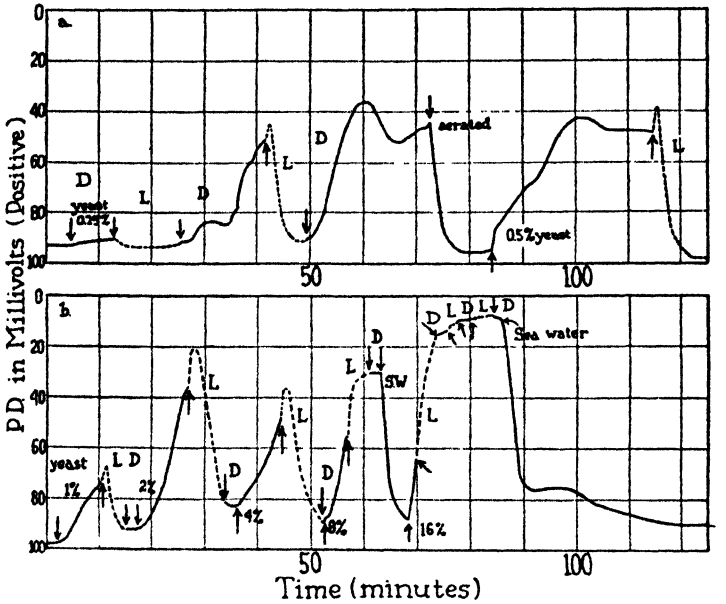

FIG. 2. The effect of light upon the P.D. of *H. ovalis*, when the cell is exposed to sea water in which active yeast cells are suspended (in percentage concentrations as marked). The P.D. starts to drift downward in the presence of only 0.25 per cent yeast, but light promptly restores it, whether given at the 90 mv. level or at 50 mv. Aeration does likewise. Good recovery also occurs on illumination in the presence of 0.5 per cent, 1 per cent, 2 per cent, and 4 per cent yeast, but with marked downward cusps preceding the recovery. This closely parallels the effect of light at low oxygen tensions (Fig. 3). In the presence of 8 per cent and 16 per cent yeast, however, light produces no recovery, although the restoration of sea water does. This failure to recover is probably not due to the exhaustion of oxygen, since light can usually overcome even the most anaerobic conditions; but to the accumulation of narcotic quantities of CO_2 in the higher yeast concentrations, which photosynthesis is unable to reduce sufficiently (*cf.* Fig. 8). Alcohol in any quantities the yeast could produce in this short exposure, is not effective.

Tracings of actual Micromax records, balanced every 3 seconds. Solid lines represent dark (*D*) and broken lines light (*L*), the arrows signifying time of change of these or other conditions. Very intense light was used, to penetrate the denser yeast suspensions.

Since the O_2 is released inside the protoplasm itself, it is directly available to the respiratory mechanisms, which probably consume their share before any O_2 diffuses out of the cell. Therefore it makes little difference in the recovery of P.D. whether the sea water is quiet during the illumination, or bubbled actively with nitrogen, or even hydrogen plus platinum black to

catalyze oxygen removal (Fig. 12). However, without such bubbling, the photosynthetically produced O_2 reaches a higher and higher concentration in the sea water, the P.D. falls more slowly on darkening, and after several illuminations, does not fall at all for some time.

Over a certain range (often 50 to 500 meter candles), light intensity determines the P.D. level reached, and to some extent, the speed of recovery. Fig. 4 shows an example of this, with fairly good reproducibility of P.D. level over an increasing, and then a decreasing, intensity series. The

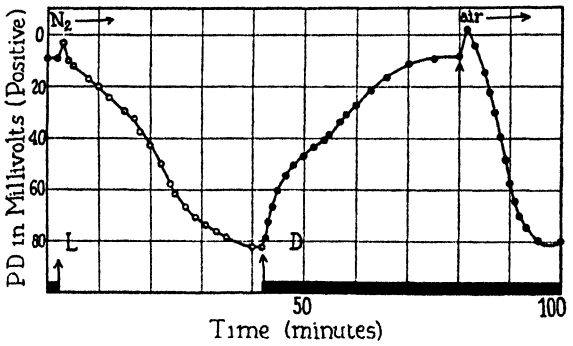

FIG. 3. Effect of light on the P.D. of *H. ovalis* in the presence of lowered oxygen tension. The sea water has been previously bubbled with nitrogen, containing 0.2 per cent O_2, and the P.D. has fallen to 10 mv. in the dark. As this nitrogen continues to bubble, the cell is then illuminated (5000 meter candles, incandescent source). There is an initial sharp decrease of P.D., followed by a steady rise until the normal P.D. of about 82 mv. is restored. The P.D. falls again rapidly in the dark to 10 mv. The bubbling of air at this point, instead of N_2, again restores the normal P.D. in the dark, after an initial depression (here to a slightly negative value). Note the marked resemblance of the recovery curves in the two cases.

Closed circles represent dark observations (*D*), open circles, light (*L*). Dark periods also indicated here by the black bars at the base of the graph. Vertical arrows indicate moments of illumination and darkening, and of admission of air.

somewhat lower levels reached on the decreasing scale may represent a lower vacuolar and protoplasmic O_2 content, as the result of the long continued N_2 bubbling, possibly aided by the increased respiration known to follow photosynthesis. In Fig. 4 the intensity was not increased sufficiently to reach normal P.D. values, but when the latter level is reached, further increase of light intensity has little further effect (Fig. 5). This is probably due to a sufficiency of O_2 to maintain the normal bioelectric properties, not necessarily to a limiting rate of photosynthesis. The experiment does not give information on this point, although Warburg manometric measurements with *Halicystis* cells do indicate that photosynthesis is also at a nearly

saturated rate in sea water at comparable light intensities, CO_2 probably becoming a limiting factor at this temperature.

A characteristic of the light response, even at lower intensities, but especially at high intensity, is the cusp which carries the P.D. downward before it starts to recover (Figs. 2, 3, and 5). This might indicate some unsus-

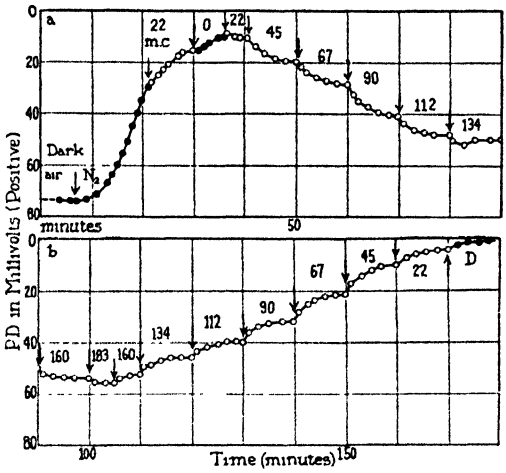

FIG. 4. Effect of light of several different intensities upon the P.D. of *H. ovalis* exposed to low oxygen tension. The dark P.D. was about 75 mv. in aerated sea water. At the first arrow, the latter was then bubbled continuously with nitrogen containing 0.2 per cent O_2, and the P.D. soon fell to about 10 mv., being only slightly retarded in its fall by weak illumination (22 meter candles). Successive increments of about 22 meter candles, however, raised the P.D. in stepwise fashion up to about 56 mv. at 183 meter candles. Corresponding decreases of intensity lowered the P.D. again, to almost zero in the dark (*D*). The lower values reached on the decreasing scale probably are due to the more complete exhaustion of O_2 from the protoplasm, both by the long continued bubbling with N_2 and the enhanced respiration of the cell following photosynthesis.

Figures represent the intensity in meter candles, as measured by a photronic meter placed as closely as possible in the position of the cell. The source was an incandescent lamp, moved along an optical bench to give the desired intensities. Arrows indicate moment of changed intensity. Adequate CO_2 was insured by bubbling the N_2 previously through a bicarbonate solution. Open circles, light; closed circles, dark.

pected anomaly of the time course of photosynthesis itself, but since an almost identical cusp is produced by the admission of air (Fig. 3), it seems to be concerned rather with the respiratory or bioelectric mechanism. The explanation may be that suggested by Gaffron,[16] that during anaerobic

[16] Gaffron, H., *Biochem. Z.*, Berlin, 1935, **280**, 337; *Naturwissenschaften*, 1937, **25**, 460, 715.

conditions there accumulate metabolites with an R.Q. greater than unity, which produce an excess of CO_2 when oxygen is first admitted (or produced within the cell). We shall see below that CO_2 and other weak acids can depress the P.D.; in fact there is strong resemblance between their effects and those of low O_2, and it may well be that the latter effect is chiefly due to increased protoplasmic acidity. (Green algae have been shown to produce acid (probably lactic) anaerobically.[17])

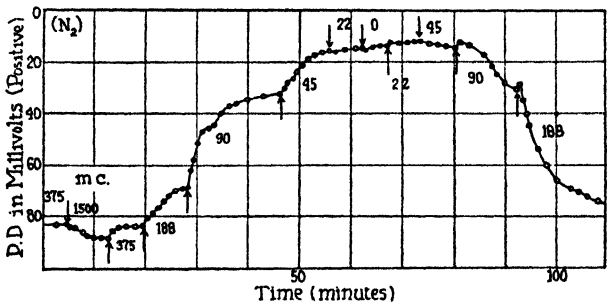

FIG. 5. Effect of varying light intensity on the P.D. of *H. ovalis* exposed to sea water with low oxygen content (continuously bubbled with nitrogen containing 0.2 per cent O_2). At the beginning of the record the cell had been exposed to this overnight, and the P.D. had dropped to 15 mv. in the dark. But 375 meter candles from an incandescent source were sufficient to restore the P.D. to 83 mv. (start of graph). This was raised only to 88 mv. by quadrupling the intensity to 1500 meter candles (higher intensities did not increase the P.D. further). The intensity was again decreased to 375 meter candles with restoration of the original value; then the intensity was halved successively to 188, 90, 45, and 22 meter candles respectively as marked. The latter two intensities were insufficient to raise the P.D. much above the dark value of 14 mv., but increase to 90 meter candles after an initial cusp, raised the level to 30 mv., and 188 meter candles to 75 mv. It is evident that most of the effective range where light controls the P.D. was in this case 50 to 400 meter candles, higher and lower intensities having little further effect.

Open circles, light; closed circles, dark. Figures refer to meter candles.

Whatever the explanation for the cusps, their opposite direction to the main recovery drift can give very unusual curves, if only short light exposures are given. Fig. 6 shows examples where the light effect is entirely negative, decreasing the P.D., and the succeeding dark effect more positive. This is characteristic only when the P.D. is low, however; the anomalies tend to disappear as the light is left on longer, or the P.D. is carried to higher values by a rapid succession of light flashes. Other cusps, with probably the same cause, will be discussed in later sections.

[17] Gaffron, H., *Biol. Zentr.*, 1939, **59**, 288.

One further aspect of short light and dark periods is shown in Fig. 7. The P.D., fluctuating though it is, centers itself about a figure roughly corresponding to the level reached if the same *energy* is given continuously. For example, 1 minute light periods of 90 meter candles, followed by 1

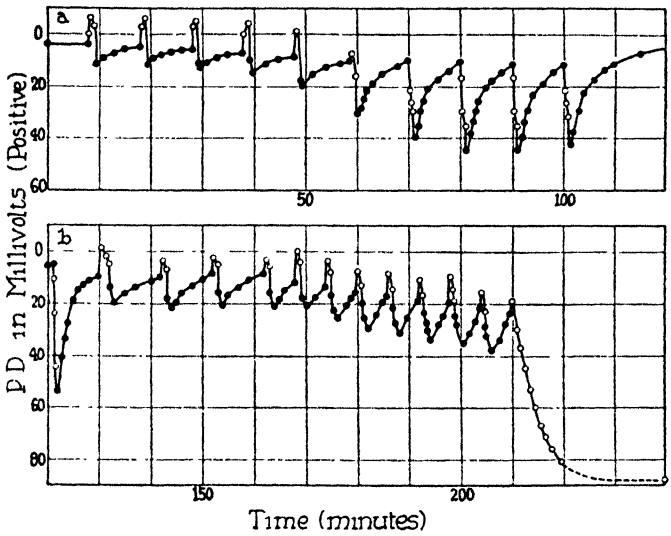

FIG. 6. Effect of short light and longer dark periods upon the P.D. of *Halicystis*, exposed to low O_2 tensions. Nitrogen had been bubbled in the dark, long enough to carry the P.D. down to 5 mv. Light (5000 meter candles from incandescent source) was then given for one 1 minute interval, followed by 9 minutes of dark. It is seen that the negative anomaly is at first the only effect of the 1 minute flash, and a reverse anomaly in the first moments of dark. Gradually however, the dark P.D. creeps up to about 10 mv. after five flashes, and after this the light flash produces strong positivity, the dark period a fall from this value.

Graph *b* continues the 1 minute exposures, after a 20 minute dark period. When the dark periods are reduced to 5 minutes each in the middle of the record, the P.D. creeps up to over 20 mv. owing to the greater total light now received. Finally the P.D. recovers its full value of 86 mv. with continuous illumination.

Open circles, light; closed circles, dark.

minute dark periods, maintain the P.D. at about the same level as 45 meter candles continuous light, under the same conditions of low oxygen tension. Thus the production of O_2 is not appreciably more efficient when the light is concentrated in these relatively long flashes. One would have to give extremely short flashes of the order of fractions of a second to obtain the extra efficiencies found by Warburg, and Emerson and Arnold. This experiment is planned for future work on *Halicystis* (*cf.* Marsh[9] on *Valonia*).

In connection with these short exposures, it should be noted that in most cases where there is any response to light at all, no appreciable induction period is observed. Even when the response is anomalous, and counter to the later drift, it usually begins as quickly as the most rapid instruments can follow; *e.g.*, photographic records of string galvanometer deflection. (But with a method which was slower in following the changes,

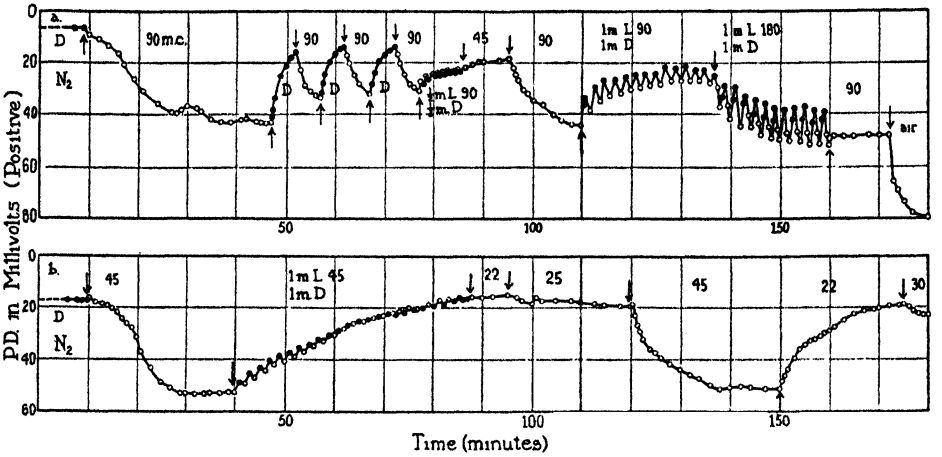

FIG. 7. Effect of alternate light and dark periods, compared with continuous illumination, upon the P.D. across the protoplasm of *H. ovalis*. The sea water was continuously bubbled with N_2 containing 0.2 per cent O_2, and the P.D. had reached a constant dark value (*D*) of about 7 mv. positive. Illumination with 90 meter candles (incandescent source) raises this to 43 mv. Three succeeding 5 minute dark periods are alternated with three light periods of 90 meter candles. The P.D. oscillates between 15 and 30 mv., with an average value of 22.5 mv. A similar average value is maintained by ½ minute alternations of the same value, but 45 meter candles continuous light, permits this to fall slowly to 20 mv. Continuous exposure, and 1 minute alternations, of 90 meter candles are repeated, with much the same effect; and alternations of 180 meter candles bring the P.D. to about the same average as 90 meter candles continuous light. Air restores normal P.D.

In graph *b*, alternations of 45 meter candles are shown in comparison with 22 and 45 meter candles steady light.

Open circles, light; closed circles, dark. Figures indicate meter candles; arrows time of changes.

the initial anomalies might give an *apparent* induction period before the main rise appeared.) Only when there is some question of injury or of toxic substances involved, as after very long anaerobic conditions, and especially when other organisms (*e.g. Ulva*) have been used to exhaust the oxygen, does an appreciable induction period appear. Then the P.D. may be practically zero, and show no instant response to light, several minutes elapsing before it even begins to drift slowly upward. The explanation for this is probably that toxic products (including H_2S) must be oxidized before the P.D. can recover. It seems significant that under these conditions, the introduction of just

a trace of atmospheric oxygen (as by merely lifting the cell out of the vessel and replacing it) often then enables light to produce an immediate and large bioelectric response, even though the P.D. has not been greatly increased by this O_2 in the dark.

Increased CO_2 Tension

It seemed possible that long exposure to low O_2, by bubbling with nearly pure N_2 gas, might scrub out most of the CO_2 from sea water and cell, to the point where low CO_2 might limit the photosynthetic rate and the evolution of O_2. CO_2 was therefore added in some cases to the N_2 stream by metering with a bubble counter,[13] or by passage through bicarbonate solution. In neither case was the recovery in light any faster or more complete than in the pure gas, indicating that sufficient CO_2 was available from anaerobic respiration, or from carbonates in cell and sea water, so that it had not become limiting (*cf.* also *Valonia*[9]).

On the contrary, indeed, when CO_2 was considerably increased, up to 15 or 20 per cent in the N_2 stream, it *inhibited* the recovery of P.D., even at high light intensities, either abolishing the response, or limiting it to the negative anomaly, without later positive drift. Since high CO_2 concentrations are known to inhibit photosynthesis, this result would not be surprising. However, this is probably not the explanation involved here; but rather, that CO_2 has a depressing effect upon the P.D. itself, quite aside from photosynthesis. Added to sea water, it lowers the P.D., even in the presence of air; and this depression begins at somewhat lower CO_2 tensions than those that inhibit photosynthesis, since light can still restore the P.D. over a limited CO_2 range. The depression can be ascribed to an effect upon ionic mobilities in the cell surface, probably due to increased acidity of the protoplasm, caused by the penetrating CO_2 (pH change of the sea water alone is not effective). The nature of this is indicated in the discussion. Recovery would of course be due to photosynthetic consumption of this CO_2.

Fig. 8 indicates some of the characteristic light effects in the presence of increased CO_2 tension. It will be noted that recovery in light only occurs if the P.D. has not been carried too far down, contrary to the situation with low O_2. If there is too much CO_2, light cannot overcome it, either because the acidity is too great to be overcome, or photosynthesis is itself inhibited. It is possibly the former, since the negative anomaly may still persist, in fact may be the only effect of light. At intermediate levels, however, complete recovery may be induced by sufficient light, often with marked cusps. This may well explain some of the larger effects in leaves, etc.

To some extent these same effects are produced by other weak acids (*e.g.*, acetic) which penetrate the cell readily. Since it is hardly likely that these

are themselves destroyed in photosynthesis, the recovery of P.D. in light must then be ascribed to the reduction of CO_2 which has been released from

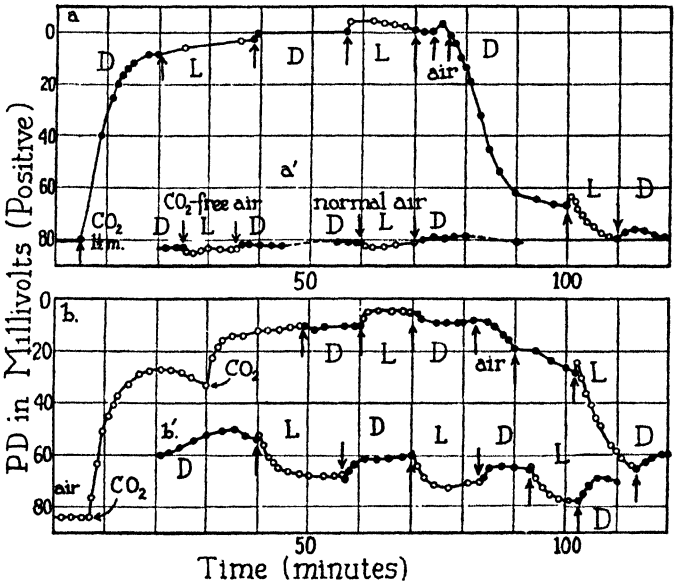

FIG. 8. P.D. across the protoplasm of *H. ovalis*, as influenced by CO_2 and light. In curve *a*, sufficient CO_2 is bubbled into the sea water to lower its pH to 5.0, and the P.D. falls to very low values, eventually to zero. Light (daylight 570 meter candles) has here little influence in preventing the fall, or restoring the P.D. (although such intensity fully overcomes low O_2 effects). Note the negative anomaly on the second exposure. Air is then bubbled in the dark, with prompt recovery of P.D. At 66 mv., the same light intensity now causes full recovery of P.D.

Compare the slight effects of light on the same cell in sea water bubbled with air, and CO_2-free air (curve *a'*).

Curve *b* indicates a similar experiment in which the P.D. is carried down, by two successive bubblings with CO_2, even under continuous illumination (750 meter candles). The negative anomaly is again shown at low P.D. values. Air is then bubbled twice, sweeping out some of the CO_2, whereupon light produces a quick rise of P.D. (after a cusp). Curve *b'* continues this graph, indicating how the P.D. is gradually driven higher on successive light exposures, which consume the CO_2 in and near the cell.

Open circles, light (*L*); closed circles, dark (*D*), arrows showing time of change.

carbonates inside these cells by the penetrating acid. Naturally the margin of acidity which can here be overcome is even less than with CO_2 alone, and it is indeed found that only moderate depressions of P.D. can be overcome. In these cases, however, the negative anomaly may often persist, as if the acidity were still further increased at first by light.

Ammonia

Since the depression of P.D. by CO_2 and the recovery in light are probably due to internal pH changes, it is fortunate that there is an independent means of sensitizing the cells to pH changes in the external medium. Normally the P.D. of *Halicystis ovalis* does not respond appreciably to acidity changes in the sea water between pH 6 and 10, especially if the sea water is well aerated after the changes, to bring the CO_2 back into equilibrium with the air. (*H. Osterhoutii* is somewhat more sensitive to pH change.) Nor is the response to light appreciably changed over this pH range. But if a little ammonia (NH_4Cl or other ammonium salt) is added to the sea water around the cells, pH changes in this medium strongly affect the P.D. Increased pH now depresses the P.D., or reverses its sign if sufficient ammonia is present; decreased pH counteracts such reversal, and raises the positive P.D. Both of these effects are due to altered dissociation of the ammonia, the free base of which readily penetrates the protoplasm, and alters the P.D. (again accompanied by an alteration of ionic mobilities in the surface; see Discussion).

When these effects were first described[12] it was remarked that light fluctuations (passing clouds, etc.) influenced the P.D. much more than normally, and it was suggested that pH changes were responsible, due to CO_2 assimilation. Further study has shown to what a remarkable extent light can control the P.D. of *Halicystis* in the presence of ammonia.

The following effects have been found:

(*a*) A given ammonia concentration produces a larger effect in the light than in the dark; the P.D. falls to a lower value, the concentration for reversal to a negative sign is less, and the negative P.D. reached is greater.

(*b*) The direction of the light effect in the presence of ammonia is the opposite of the small normal one, or the main recoveries from low O_2 or high CO_2; light now makes the P.D. *less* positive (or more negative), instead of *more* positive.

(*c*) There are, however, even more striking anomalies in the general time course, by which the initial cusps, noted in the previous treatments, become tremendously magnified. This again suggests an initial acidity change in the opposite direction to that generally assumed in photosynthesis. And there are corresponding anomalies in the darkening response.

Figs. 9 and 10 show many of these characteristics, including the hysteresis which is characteristic of ammonia treatments; a smaller ammonia concentration is required to maintain a reversed P.D. than to induce it originally. Similarly, a concentration which is not quite sufficient to reverse the P.D.

alone, often maintains it in the dark after light has aided in the reversal. This hysteresis even extends to the anomalous cusp which drives the P.D.

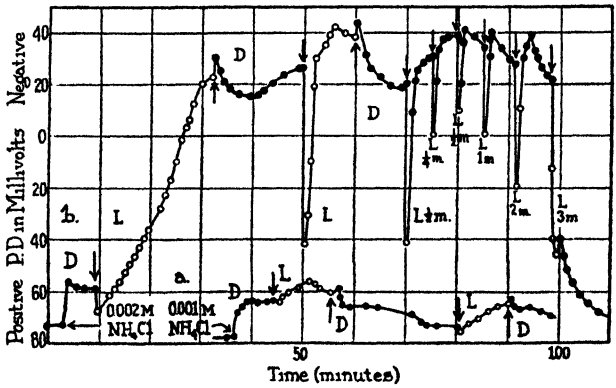

FIG. 9. Effect of light on the P.D. across the protoplasm of *H. ovalis*, when the cell is exposed to sea water containing ammonia below the threshold for reversal of P.D. The addition of 0.001 M NH_4Cl to the sea water at pH 8.0 lowers the P.D. to about 65 mv. Illumination (2100 meter candles, incandescent source) then produces a slight increase after which the P.D. drifts downward, and in 10 minutes reaches a rather constant value at about 60 mv. Darkening at this time produces a small counter-cusp, after which the P.D. rises to about 73 mv. Succeeding light periods decrease the P.D., but always after an initial cusp which increases the value. Converse cusps occur on darkening, becoming more conspicuous as the P.D. falls to lower levels.

Then in curve (*b*) 0.002 M NH_4Cl is added. The P.D. falls to 58 mv. (after a cusp) but shows no further sign of reversing in the dark. When illuminated however (3000 meter candles), after an initial increase, the P.D. rapidly falls and reverses to about 22 mv. negative. Darkening at this point produces a further negative cusp, followed by a decrease and rise, characteristic of the P.D. when first reversed by ammonia. A second illumination produces a very striking cusp which causes recovery to 40 mv. positive before reversing back to 40 mv. negative.

After a 10 minute dark period, a half-minute light flash produces another large cusp to 40 mv. positive, with prompt return to negative P.D. Successive short flashes repeat this effect, the cusp becoming greater with longer durations and finally, at 3 minutes, being carried so far positive that darkening intervenes before the later light effect can induce reversal, and the P.D. recovers its normal positive sign. This hysteresis is characteristic of ammonia near the threshold. The acid gush first produced by light causes recovery before the later alkaline drift can cause reversal (as in the first part of *b*).

Open circles, light periods (*L*); black circles, dark (*D*). Arrows indicate beginning and end of illumination (only beginning shown with short flashes, whose duration is indicated by figures).

more positive during the first moments of illumination, before the negative influence predominates. Fig. 9 shows an example: 0.002 M NH_4Cl is unable to reverse the P.D., until aided by 20 minutes illumination. The negative

P.D. is then in turn maintained in the dark, until a *very short* light exposure induces recovery. The light is then turned off *during* the positive cusp. In the dark the P.D. starts toward negativity, but is too far positive to continue, and recovers. Naturally the ammonia concentration must be

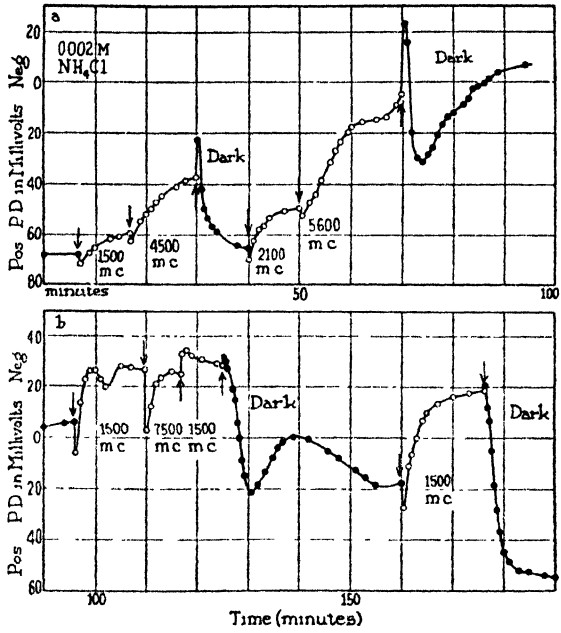

FIG. 10. Effect of several light intensities on the P.D. across the protoplasm of *H. ovalis*, exposed to subthreshold ammonia concentration. This is the same cell used in Fig. 9, which then stood overnight in 0.002 M NH_4Cl, without reversal of P.D.

Illumination with increasingly higher light intensities, as marked, decreases the P.D.; 5600 meter candles carry it so low that the P.D. reverses on later darkening, first with a large cusp, and later more permanently. In *b*, light carries this negative P.D. to still higher values, but only after initial cusps in the opposite direction. The slow recovery in the dark indicates that the ammonia is very close to threshold after the continued illumination. More rapid recovery follows the last, shorter light period. Note the smaller upward cusps when darkening succeeds lower light intensities.

Open circles show light periods, black circles, dark, with arrows indicating beginning and end of periods. Incandescent source on optical bench giving light intensities as marked, in meter candles, metered with photronic cell.

well adjusted, close to the threshold, to show this effect, but it illustrates how light can be used to throw the P.D. permanently in either direction. A somewhat analogous effect was described in a previous paper,[18] for the concurrent action of ammonia and current flow.

[18] Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 867.

Such sensitization to light by a substance often found naturally in the vacuole of *Halicystis* cells,[12] and tolerated without injury for long periods, evidently gives important clues to the changes of pH occurring during illumination, both inside and outside the cell. On the whole these agree with the expected rise of pH by consumption of CO_2, causing increase of undissociated ammonia, and fall or reversal of P.D. as a result. Two questions, however, remain: (1) is this change effective inside the protoplasm, or outside it; and (2) what causes the "cusps" in the opposite direction during the first moments of illumination (as well as the reverse cusps on darkening)?

As to whether the pH changes are effective inside or outside the cell, a partial answer is given by experiments in which the sea water is kept buffered (*e.g.*, by borate) during exposure to ammonia and light. The regular ammonia effect still occurs in the dark, indeed at a somewhat lower concentration of NH_4Cl than in ordinary sea water. This is probably because the borate keeps the sea water next the cell better buffered, and it does not become as acid when the ammonia penetrates the cell leaving HCl behind. But illumination does not now aid the ammonia effect very well, probably because the better buffering again prevents as great pH changes outside the cell due to CO_2 consumption. On the other hand, the anomalous cusps, which carry the P.D. more positive during the first moments of illumination, still persist in the presence of borate buffer. In fact they may even become more conspicuous because the ammonia cannot later enter as rapidly; and the same is true of the contrary cusps produced on darkening.

This makes it seem likely that the regular negative drift with ammonia plus light is due to the expected rise of pH outside the cell (due to photosynthetic utilization of CO_2). This shifts the ammonia dissociation so that more free base may enter the cell. On the other hand the initial anomalies are due to increased acidity largely restricted to the protoplasm itself, and counteracting the ammonia which has entered there. It is probably capable of lowering the pH outside, only if CO_2 is released and able to move out.

The converse dark effect, of decreased acidity preceding respiration, might be due to the dark consumption of CO_2 by a mechanism prepared for it in the light.[19, 20]

These explanations of the anomalies in terms of acidity changes have gained confirmation by direct pH measurements, as described below.

[19] McAlister, E. D., *J. Gen. Physiol.*, 1938–39, **22**, 613.

[20] Ruben, S., Hassid, W. Z., and Kamen, M. D., *J. Am. Chem. Soc.*, 1939, **61**, 661.

A previous paper[14] has shown that a glass electrode in direct contact with the leaves or thallus of various terrestrial and aquatic plants, rapidly responds to the pH changes resulting from respiration or photosynthesis. Diffusion is so rapid across the few microns of solution between cells and electrode that the response to illumination is almost instantaneous, within the period of the galvanometer employed. The normally expected drifts toward acidity in the dark due to CO_2 production, and toward alkalinity during photosynthesis, are always shown, in a variety of tissues. But in many cases there are also anomalies during the first moments of illumination or darkening, *i.e.* toward acidity before the alkaline drift in the light, and toward alkalinity before the acid drift in darkness.

These anomalies have been found so generally in leaves, *Ulva*, and suspensions of unicellular algae, that they could well be assumed to account for the bioelectric anomalies in *Halicystis*. It was of interest, however, to see whether such anomalies could be actually demonstrated in *Halicystis* itself. The problem of making close contact with the spherical cell surface was a little troublesome, but was solved by using a cup-shaped glass electrode, very like the early design of Tookey-Kerridge. The somewhat resilient cell of *Halicystis* was readily pressed into close contact with this cup (the rest of the electrode being paraffined); the cup could also be used to support an impaled cell for concurrent bioelectric measurements. Circuits were so arranged that changes in the glass electrode and bioelectric circuits could be recorded simultaneously on the same photographic paper, without interference with each other.

When the cell was illuminated, the glass electrode record often displayed a small cusp toward greater acidity before the alkaline drift set in. This happened even when there was little or no bioelectric effect of light; *i.e.*, when the P.D. was normal. A greater response was sometimes shown when the cell was exposed to ammonia. On the other hand when the sea water was better buffered, little or no change of pH could be detected, even though the bioelectric response might be showing large cusps. This indicated the independence of the two circuits, but also, more significantly, that the acid gush is essentially an internal affair, within the protoplasm, and appears outside only if the latter is a poorly buffered solution. (Effects with leaves, etc., are also best shown in distilled water, or unbuffered sea water.)

The *external* appearance of the acid gush is probably dependent upon the decomposition of internal carbonate, and the release of CO_2 to the exterior. Probably the rather slight acid anomalies shown by the glass electrode with *Halicystis* are due to the very thin protoplasmic layer, with small carbonate reserves compared to the vacuole and sea water. The appearance of even a slight cusp is significant under these conditions.

Ammonia Plus Low O_2

Even in the dark, this combination has been described[13] as giving complex time courses; the addition of light as a third variable gives, as might be expected, still greater complexity. Not only cusps, but three and four peaked curves have been obtained, which rise, fall, and reverse in succession. Fig. 11 gives an example. The behavior evidently depends upon the time sequence of anomalous acid gush, oxygen production, regular CO_2 consumption, and the ammonia hysteresis. No doubt CO_2 and other variables

could be introduced to complicate the picture further—just as many of these factors are probably present in leaves, etc., to give the complex curves

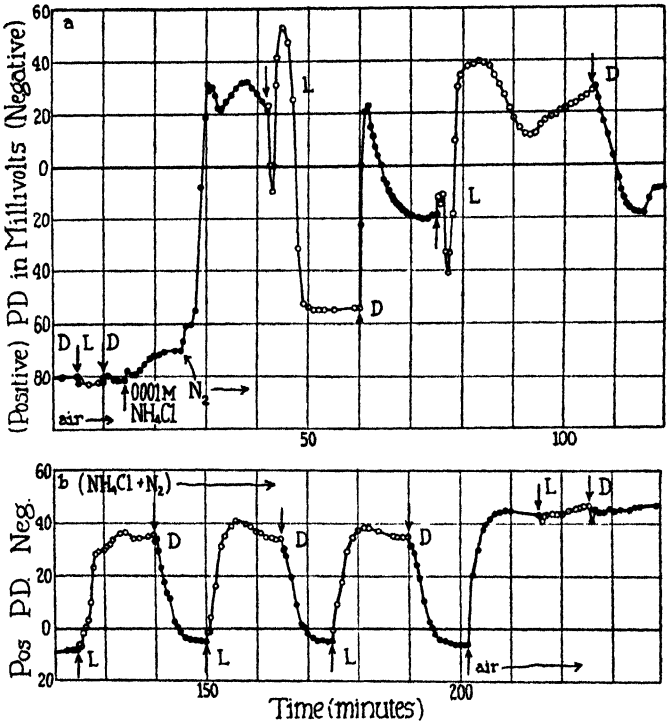

FIG. 11. Effect of light on the P.D. of *H. ovalis*, when exposed to low concentrations of NH_4Cl, in the presence of low O_2 tension. A small normal light effect is first seen. Then 0.001 M NH_4Cl is added to the sea water, depressing the P.D. to 70 mv., but with no further sign of reversal. However, when N_2 (plus 0.2 per cent O_2) is then bubbled, the P.D. quickly reverses, with characteristic negative cusps. When these are largely over, daylight is given (1000 meter candles). The P.D. recovers positivity, after a complex curve with one small and two large cusps. Darkening gives a pronounced negative cusp with partial recovery; and light a final negativity *after four cusps*.

Thereafter, in curve *b*, light merely made evident the negative P.D. characteristic of ammonia, while dark reduced this to the low positive value characteristic of low oxygen. Finally air was admitted, having much the same effect as light; and succeeding light responses were negligible, ammonia now thoroughly dominating the P.D. (Later bubbling with N_2, not shown, gradually restored the light effects of the first part of curve *b*.)

there observed. The fact that the very simple and slight light response in normal *Halicystis* can be so enormously complicated by experimental control, is perhaps the chief lesson to be drawn from Fig. 11.

Other Light Effects

In the course of other treatments, light has been found to have effects comparable to those here included. Perhaps the most surprising was in the presence of unbalanced NaCl, the effects of which are markedly enhanced by light. Since this agent acts somewhat like ammonia, an increase of alkalinity is probably involved.

Other light effects have been found in the presence of various metabolic stimulants and inhibitors, and will be described in a later paper.

DISCUSSION

It is now desirable to inquire more closely into the causes of the light effects.

Since light does not greatly alter the normal P.D. of *Halicystis*, we may probably rule out any direct effect of light upon the normal permeability of the cell (as by altering the mobility and solubility of ions in the surface). Nor, for the same reason, does it appear to have any immediate effect, at least, upon the sources of potential energy, such as the gradients of ions, internal or external, which by their differing mobilities in the two surfaces, give rise to the observed steady P.D. Of course it must be granted, since *Halicystis* is an autotrophic plant, dependent eventually on photosynthesis for its entire substance and existence, that light is ultimately responsible for all the phenomena which it displays: for both surfaces and ion gradients alike. Without light during their previous growth period, the cells simply would not be here to experiment with. But with reference to any short illumination periods, and even the normal day and night fluctuations, no great effect upon the electrical system is produced. It is already set up and running, with fairly stable surfaces and internal ion sources, not immediately disturbed by light or dark.

However, this rather stable normal P.D. can be altered or sensitized by certain treatments, as we have seen, and then light may have a powerful influence, by acting with or against these treatments. Fortunately, something quite definite can now be said about some of these changes, and incidentally about the source of P.D. itself.

This grows out of recent work in this laboratory upon the substitution of various anions for the chloride which predominates in the sea water and sap. (Most of the previous attention had centered upon cation substitution.) This will not be documented here, since it will be more fully presented in another paper. But a brief outline is necessary. As long appreciated in

Valonia[21] and recently shown in *H. Osterhoutii*,[22] the mobility of Cl^- ion in the outer surface of *H. ovalis* also is greater than that of Na^+. Sea water can thus contribute to the positive P.D., (assuming it remains less concentrated in the protoplasm); and the P.D. is actually decreased by dilution of the sea water, although the extent of this change is limited. Br^- resembles Cl^-, although it is not so mobile. But the situation is quite different with all the other anions which have been tried, both inorganic and organic: nitrate, sulfate,[13] formate, acetate, propionate, butyrate, lactate, pyruvate, glutamate, and others. When sodium salts of these replace the NaCl of sea water, the P.D. is greatly decreased, or even reversed on their full substitution (with some Mg, Ca, and K present to maintain physiological balance). Such an anion-substituted sea water also shows a reversed dilution effect (dilute solution more positive, instead of more negative as with chlorides) indicating that the mobility of Na^+ in the protoplasmic surface is now greater than any of these anions (which are all much alike in effect).

The effect is similar when such sea waters (nitrate, formate, etc.) are perfused in the vacuole, except that, being applied internally, the P.D. is increased, from the normal 80 mv., up to 100 mv. or more (the first clear case of appreciable experimental increase of the *Halicystis* P.D.). But, even with very thorough perfusion, such increase is not at all as great as the decrease produced by external exposures to the same solution. This experiment directly demonstrates, therefore, the long postulated difference between the inner and outer surfaces of the protoplasm, the inner being less sensitive to the substitution of these anions than the outer. (It is also less sensitive to K^+ ions, and to the dilution of the natural sap or of sea water.) Consequently we may expect it to contribute less to the observed potential than does the outer surface.

We may now postulate a high concentration of less mobile anions in the aqueous protoplasm (organic acids, amino acids, proteins), plus more mobile cations (K, Na) making a total equivalent concentration approximately equal to the sea water salts. (This would be in osmotic equilibrium with the sea water, assuming the carbohydrates to be mostly starches, etc., rather than sugars.) These will set up a large outwardly directed potential across the outer surface, and a smaller inwardly directed potential across the less responsive inner surface (the vacuole, like the sea water, being nearly free of such less mobile anions). The two potentials in algebraic sum would give the observed P.D. across the entire protoplasm.

[21] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 445.
[22] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 75.

Any change in this P.D. would then be due to either: (*a*) a change in the equivalent concentration of the protoplasmic ions themselves (or in their charge if amphoteric); or (*b*) a change in the properties of the surfaces, altering the partition or mobilities of the ions in them. The first change is not readily accessible to experimental test, but could of course occur as the result of decreased or increased metabolic production of such ions (*e.g.*, lactic acid, pyruvic acid, etc.) under low oxygen tension; a change of charge could result from the penetration of acids (CO_2) or bases (NH_3) under experimental conditions such as have been described in this paper. A change in the surfaces could, however, be tested directly by applying externally or in the vacuole such solutions as nitrate and formate sea waters, which normally give large potential changes. Any changes in their effect which paralleled the change of P.D. might indicate that the response of the surface to internal ion sources was also being altered in the same way.

As a matter of experimental fact, it is almost invariably this change which occurs when the P.D. alters. Such a concurrent alteration was shown in a previous paper[13] during exposures of *Halicystis* to low O_2 tension, using in that case the response to KCl as a test of the surface response. The KCl test is not ideal, since the K effect is transient, and there are also changes after the exposure which have to be allowed for. Nitrate, and similar sea waters are much more satisfactory, giving lasting and reproducible alterations of P.D. They give even more strikingly the same kind of picture: as the P.D. falls due to low O_2, then *concurrently and proportionally*, the response to these sea water substitutions decreases, until, when the P.D. is lowest, there is also practically no response to external substitutions (or to dilutions of any of the sea waters, including chloride sea water). It seems reasonable to conclude that the P.D. has likewise fallen because the cell surface no longer distinguishes between internal anions and cations, any more than it demonstrably does between external ones. Since the electrical resistance also rises greatly,[13] the mobility of ions has evidently been greatly decreased, as well as equalized in the cell surface.

This resistance rise is emphasized, since it might be thought that the protoplasm was "injured" by the low O_2 tension, becoming like a dead cell, and very permeable to all ions. Quite the contrary is evidently the case, the protoplasm becoming much *less* permeable to ions. Thus the direct current resistance rises from the normal value of 100 or 200 ohms, to several thousand ohms, per sq.cm., of cell surface. Along with this resistance rise, very large polarization curves appear at make and break of the measuring current, due to a very large capacity.

Then on restoration of oxygen (whether by aeration or by light) the P.D.

recovers promptly, and with it, *pari passu*, the response to nitrate and similar sea waters (as well as to dilution of normal sea water). The electrical resistance also falls, indicating an increased permeability to ions.

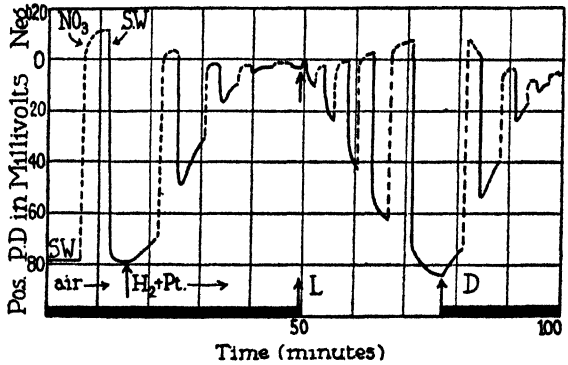

FIG. 12. Graph indicating the loss of ionic response in *Halicystis ovalis* under the influence of low oxygen tension, and its restoration by light. The P.D. in aerated sea water is first shown (*ca.* 78 mv. positive); then the interrupted line indicates the great change produced by bathing the cell in nitrate sea water (NO_3) (artificial sea water in which all the chloride and sulfate have been replaced by nitrate). The P.D. is promptly altered by about 90 mv., becoming about 10 mv. negative. It would remain at this level for 30 minutes or more in this solution, but promptly recovers its normal value on restoration of sea water (S.W., shown by the solid line). Then the cell is bathed in a sea water previously bubbled with hydrogen, plus some suspended platinized asbestos, to hasten the disappearance of dissolved oxygen (H_2 + Pt). The P.D. soon begins to fall. At about 70 mv., sea water is quickly replaced with nitrate sea water (broken line), also well bubbled with hydrogen, plus platinized asbestos. The P.D. reverses as before, but to a lower negative value, and this begins to fall toward zero. Oxygen-free sea water is then restored (solid line), and the P.D. recovers only to about 50 mv., and rapidly falls toward zero. Three more substitutions with oxygen-free nitrate sea water follow, as the P.D. falls, with a correspondingly lessened effect each time. In other words the nitrate effect disappears along with the falling P.D.

At the arrow, light is then given; the solutions still remaining oxygen-free; but the O_2 released photosynthetically within the cell causes a rapid restoration of P.D. Along with this the nitrate effect reappears (interrupted lines). Finally the cell is again darkened, and both the P.D. and the nitrate effect disappear. This suggests the cause for the light effects, in restoring the cell surface to its normal discrimination between different ions, which are probably responsible for the P.D. itself.

The graph is an actual tracing of an automatic potentiometer (Micromax) record. Balance is made every 3 seconds, giving a practically continuous record of the P.D. changes. Dark periods here represented by black blocks at bottom of figure, light period by their absence.

Fig. 12 gives an example of this type of experiment, with lowered O_2 tension. Very similar results have been obtained with high CO_2, and with

ammonia, the surface losing its power to respond to nitrate sea water, etc., in both cases. The only difference seems to be that CO_2 appears to affect both surfaces, so that the P.D. remains low or nearly zero, while NH_3 apparently alters the outer surface while leaving the inner to display its own potential (further experiments need to be done on the latter, however, by perfusion). In both cases again, light only acts as it opposes or aids these agents in their effects upon the ionic discrimination of the cell surface.

In what manner the surface is altered by these varied agents, and what clues these alterations give as to the nature of the cell surface, we may hope that future work may solve. Acidity changes within the protoplasm are strongly suggested, whether due to penetrating acids and bases, or to acid production in anaerobic or otherwise altered metabolism. In any case we may conclude that the surface is remarkably labile, not only in response to unusual agents such as guaiacol,[23] but to natural metabolic changes in respiration and photosynthesis.

Granted the validity of the hypothesis as to the source and nature of the P.D. in *Halicystis*, most of the light effects fit well with the conventional effects of photosynthesis itself: O_2 production, and CO_2 consumption, with its resultant pH changes. No essentially new photosynthetic hypothesis has to be introduced except in respect to the initial anomalies, which are ascribed to an "acid gush" (and alkaline gush on darkening). Even these, however have acquired new standing as the result of the recent findings of Gaffron,[16] McAlister,[19] Ruben, Hassid, and Kamen,[20] and others as to unusual events during the first moments of illumination or darkening. It is even possible that the bioelectric changes, being intrinsically very rapid (because based upon events in the protoplasm itself, before they can be detected outside) may give new information on these important points.

SUMMARY

The effects of light upon the potential difference across the protoplasm of impaled *Halicystis* cells are described. These effects are very slight upon the normal P.D., increasing it 3 or 4 per cent, or at most 10 per cent, with a characteristic cusped time course, and a corresponding decrease on darkening.

Light effects become much greater when the P.D. has been decreased by low O_2 content of the sea water; light restores the P.D. in much the same time course as aeration, and doubtless acts by the photosynthetic production of O_2. There are in both cases anomalous cusps which decrease the

[23] Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 707.

P.D. before it rises. Short light exposures may give only this anomaly. Over part of the potential range the light effects are dependent upon intensity.

Increased CO_2 content of the sea water likewise depresses the P.D. in the dark, and light overcomes this depression if it is not carried too far. Recovery is probably due to photosynthetic consumption of CO_2, unless there is too much present. Again there are anomalous cusps during the first moments of illumination, and these may be the only effect if the P.D. is too low.

The presence of ammonium salts in the sea water markedly sensitizes the cells to light. Subthreshold NH_4 concentrations in the dark become effective in the light, and the P.D. reverses to a negative sign on illumination, recovering again in the dark. This is due to increase of pH outside the cell as CO_2 is photosynthetically reduced, with increase of undissociated NH_3 which penetrates the cell.

Anomalous cusps which first carry the P.D. in the opposite direction to the later drift are very marked in the presence of ammonia, and may represent an increased acidity which precedes the alkaline drift of photosynthesis. This acid gush seems to be primarily within the protoplasm, persisting when the sea water is buffered. Glass electrode measurements also indicate anomalies in the pH drift.

There are contrary cusps on darkening which suggest temporarily increased alkalinity.

Even more complex time courses are given by combining low O_2 and NH_4 exposures with light; these may have three or more cusps, with reversal, recovery, and new reversal.

The ultimate cause of the light effects is to be found in an alteration of the surface properties by the treatments, which is overcome (low O_2, high CO_2), or aided (NH_4) by light. This alteration causes the surface to lose much of its ionic discrimination, and increases its electrical resistance. Tests with various anion substitutions indicate this, with recovery of normal response in the light.

A theory of the P.D. in *Halicystis* is proposed, based on low mobility of the organic anions of the protoplasm, with differences in the two surfaces with respect to these, and the more mobile Na and K ions.

THE THEORETICAL RESPONSE OF LIVING CELLS TO CONTACT WITH SOLID BODIES

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We have chosen the above title for this analysis since the following discussion is an extension of an article by W. O. Fenn with the same title.[1]

Fenn's theoretical analysis of the behavior of a hypothetical fluid cell in contact with a flat solid surface was an attempt to solve the problem without using the mathematical fiction of a "surface tension." From consideration of the free surface energy of the cell, he showed that conditions of equilibrium between a cell settling and spreading on a plane surface can be predicted, completely, in terms of the contact angle between cell and surface. Using the same method of attack he endeavored to show that in general, when a cell spreads over a solid particle (ingestion of the particle by the cell) all equilibrium conditions could be predicted from a knowledge of the magnitude of the contact angle alone. Unfortunately, "even with the expert assistance of a professional mathematician" he was not able to arrive at a general solution so that he limited himself to a special case, in which the cell's diameter was four times the magnitude of the particle's diameter, and discussed this in detail. He concluded that the particle cannot be completely ingested unless the surface tensions at the interfaces are such that the cell in the same environment can spread to infinity when placed on a flat surface of the same substance as the particle.

In order to simplify the analysis of the general case, about to be considered, we have reviewed Fenn's discussion of the behavior of a perfectly fluid cell in contact with a plane surface by means of a simplified solution. Using the same notation adopted by Fenn we will assume the existence of a spherical cell C about to settle on a plane glass surface G, both completely immersed in plasma P. Distortions in the cell due to the gravitational field are here considered negligible.

We wish, as in Fenn's solution, to consider the various positions that such a cell will assume as it settles and spreads over the glass surface. Fig. 1 shows the position of the cell in the act of spreading with the conventional

[1] Fenn, W. O., *J. Gen. Physiol.*, 1921–22, **4**, 373.

surface tensions acting at the circumference of contact between cell and surfaces indicated. These surface tensions are not to be considered as actual forces. Treatment of them as such is very likely to lead to fallacious conclusions.

The original spherical cell is assumed to have radius r_0. In the act of spreading, the spherical cell segment of height h (Fig. 1) and glass-cell contact area πa^2, has a total area

$$x = 2\pi r^2(1 - \cos\theta) = 2\pi r h$$

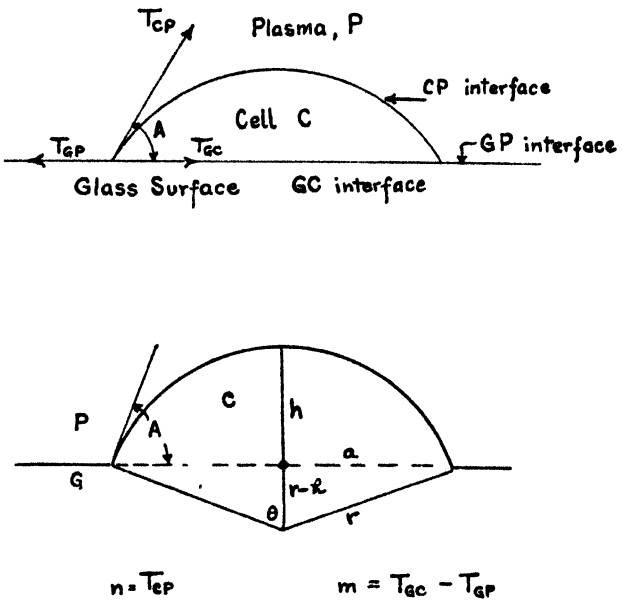


FIG. 1

composed of two parts, its curved CP interface and its circular GC interface. Since from Fig. 1, $r = \dfrac{h^2 + a^2}{2h}$ it follows that $x = \pi(h^2 + a^2)$. The surface energy of the cell after having been reduced to the form of a spherical segment is therefore

$$E = n\pi(h^2 + a^2) + m\pi a^2$$

where $n = T_{CP}$ and $m = +T_{GC} - T_{GP}$, the respective surface tensions at the interfaces indicated by the subscripts.

In order to investigate under what conditions the surface energy is a minimum, we must differentiate E with respect to h, the changing height of the cell as it spreads over the surface G. In order to do this we must

express the radius a of the area of contact of Fig. 1 in terms of h. The volume of the spherical segment is at all times equal to the original spherical volume of the cell. Then

$$\frac{4}{3}\pi r_0^3 = \frac{\pi}{3} r^3 (1 - \cos\theta)^2 (2 + \cos\theta).$$

Taking the radius r_0 of the original spherical cell as unity and substituting the values of $\cos\theta$ as obtained from Fig. 1 we obtain

$$a^2 = \frac{8 - h^3}{3h}$$

Hence

$$E = \pi n h^2 + \pi(n + m)\left(\frac{8 - h^3}{3h}\right)$$

which upon differentiating with respect to h and equating to zero gives the equilibrium values of h for various values of $\frac{m}{n}$

$$h^3 = \frac{4(n + m)}{2n - m} = \frac{4\left(1 + \frac{m}{n}\right)}{2 - \frac{m}{n}}.$$

From this equation knowing m/n we can calculate the various equilibrium values attained by the cell in terms of its height h.

The contact angle between cell and glass surface as shown in Fig. 1 and used in this solution is the angle $A = \theta$, such that

$$\text{Cos } A = \cos\theta = \frac{r - h}{r} = \frac{4 - 2h^3}{4 + h^3}.$$

After substituting the previously obtained values of h in terms of $\frac{m}{n}$, one finds that the contact angle is determined by

$$\text{Cos } A = -\frac{m}{n}.$$

This relation describes the position of the cell completely with respect to the plane surface for all values of h.

Thus, when $\frac{m}{n} = +1$, $\cos A = -1$, $A = 180°$, and the cell is a free sphere of diameter $h = 2$ units. When $\frac{m}{n} = 0$, $\cos A = 0$, $A = 90°$, the

cell is a hemisphere of radius $h = 1.26$ units. When $\frac{m}{n} = -1$, $\cos A = +1$, $A = 0°$, and the cell has spread to infinity; in reality until it has formed a monomolecular layer. Only values of $\frac{m}{n}$ between $+1$ and -1 can produce finite spreading and the magnitude of the contact angle A for a given value of $\frac{m}{n}$ describes the position of equilibrium of the cell with respect to the horizontal plane.

It follows that the spreading of the cell is favored by an increase in the value of $-\frac{m}{n}$; that is, by a decrease in contact angle. If for a given value of $-\frac{m}{n}$ an equilibrium contact angle has been attained as a cell spreads over a plane, then a decrease in contact angle thereafter implies that an increase in the value of $-\frac{m}{n}$ has been obtained either by increasing $-m$ or decreasing n. To promote cell spreading we could increase $-m$. This can be accomplished by changing the character of the surface G, perhaps by depositing on the glass surface a monomolecular layer of a substance that changes the values of these interfacial tensions; *i.e.*, "sensitizing" the surface.

Using the same analysis as outlined above we may proceed to calculate the relative surface energy of a spherical cell at various stages of ingestion of a spherical particle. It will be shown that in general one only need know the variation of the magnitude of the contact angle in terms of $-\frac{m}{n}$ to be able to predict the depth of penetration of the particle into the cell for any given ratio of particle and cell diameter.

In Fig. 2 let C be a cell of radius r and G the particle of radius g. G is represented as partly ingested by the cell, resulting in a contact angle A between the cell-plasma (CP) interface and cell-glass (CG) interface. The volume ingested (shaded area) is composed of two spherical segments of total volume

$$\frac{\pi}{3} r^3(1 - \cos \varphi)^2(2 + \cos \varphi) + \frac{\pi}{3} g^3(1 - \cos \theta)^2(2 + \cos \theta)$$

where

$$r \sin \varphi = g \sin \theta. \tag{1}$$

If the original volume of the cell is $\frac{4}{3}\pi r_0^3$, then through the progressive intrusion of G, a cell of radius $r > r_0$ is being produced. The original volume $\frac{4}{3}\pi r_0^3$ has therefore been increased by the sum of two spherical segments, such that

$$\frac{4}{3}\pi r_0^3 = \frac{4}{3}\pi r^3 - \left[\frac{\pi}{3} r^3(1 - \cos\varphi)^2(2 + \cos\varphi) + \frac{\pi}{3} g^3(1 - \cos\theta)^2(2 + \cos\theta)\right] \tag{2}$$

As before, we will let the magnitude of r_0, the radius of the undistorted cell, be unity. The total surface energy of the system may then be expressed as

$$E = 2\pi n r^2(1 + \cos\varphi) + 2\pi m g^2(1 - \cos\theta) \tag{3}$$

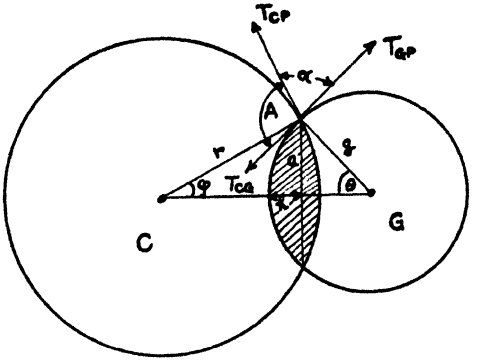


FIG. 2

Since we are interested in establishing the depth to which the particle sinks into the cell, we will choose θ as the independent variable and investigate the condition for minimal surface energy at equilibrium, when $\frac{dE}{d\theta} = 0$. The result of differentiating relations 1, 2, and 3 gives equations 4, 5, and 6.

$$\frac{dr}{d\theta}\sin\varphi + r\cos\varphi\frac{d\varphi}{d\theta} = g\cos\theta \tag{4}$$

$$0 = [4 - (1 - \cos\varphi)^2(2 + \cos\varphi)]r^2\frac{dr}{d\theta} - r^3\sin^3\varphi\frac{d\varphi}{d\theta} + g^3\sin^3\theta \tag{5}$$

$$0 = 4\pi n r\frac{dr}{d\theta}(1 + \cos\varphi) - 2\pi n r^2\sin\varphi\frac{d\varphi}{d\theta} + 2\pi m g^2\sin\theta \tag{6}$$

After eliminating $\frac{d\varphi}{d\theta}$ and $\frac{dr}{d\theta}$ we get

$$r^2 g\frac{\sin\varphi\cos\theta}{\cos\varphi} - \frac{m}{n} r g^2\sin\theta = g^3\sin^3\theta + r^3 g\frac{\sin^3\varphi\cos\theta}{\cos\varphi}. \tag{7}$$

Substituting values of r from equation (1) we get

$$\sin\theta\left[\cos(\theta + \varphi) - \frac{m}{n}\right] = 0 \tag{8}$$

Hence

$$\cos(\theta + \varphi) = \frac{m}{n}. \tag{9}$$

Since from Fig. 2, $\theta + \varphi = \pi - A$, where A is the contact angle,

$$\cos A = -\frac{m}{n}. \tag{10}$$

Just as in the case of the cell spreading over the plane surface, so here the cosine of the contact angle A (Fig. 2) describes completely the position of the particle with respect to the cell as it is progressively ingested, through spreading of the cell over the surface of the particle.

Then when $-\frac{m}{n} = -1$, $\cos A = -1$, $A = 180°$, the cell is a free sphere. When $-\frac{m}{n} = 0$, $\cos A = 0$, $A = 90°$. Under the circumstances no information can be obtained about the penetration of particle into the cell, unless the ratio of the particle to cell diameter is known. If, however, as in Fenn's case, the ratio of the radii is assumed as $r_0/g = 4$, then the depth of penetration of the particle into the cell for $A = 90°$ is $0.79g$. The particle has penetrated to nearly $\frac{1}{5}$ of its diameter. The particle is completely immersed in the cell when $-\frac{m}{n} = +1$, $A = 0$ at equilibrium. This is also borne out by Fenn's special solution, $r_0/g = 4$.

The preceding analysis gives complete information on the equilibrium configuration of the system, for any relative sizes of cell and particle but does not tell anything about the equilibrium or non-equilibrium values of the surface energy. This lack can be remedied if the cell diameter is large in comparison with that of the particle, $r_0 > g$, in which case one can obtain an explicit approximate expression for the dependence of the surface energy E on the contact angle A or on θ, the angle measuring the depth of penetration of the particle.

Let $g/r_0 = \lambda$, $r/r_0 = \alpha$, then from equation (7)

$$\alpha^2 = 1 + \frac{\lambda^2}{4}(1 - \cos\theta)^2(2 + \cos\theta) + \frac{\alpha^2}{4}(1 - \cos\varphi)^2(2 + \cos\varphi).$$

From equation (1), $\alpha \sin\varphi = \lambda \sin\theta$.
Eliminating φ between these two equations we get

$$\alpha^2 = 1 + \frac{\lambda^2}{4}(1 - \cos\theta)^2(2 + \cos\theta)$$
$$+ \frac{\alpha^2}{4}\left[1 - \sqrt{1 - \left(\frac{\lambda \sin\theta}{\alpha}\right)^2}\right]^2\left[2 + \sqrt{1 - \left(\frac{\lambda \sin\theta}{\alpha}\right)^2}\right]$$

from which, neglecting powers of λ higher than the fifth,

$$\alpha = 1 + \frac{\lambda^3}{12}(1 - \cos\theta)^2(2 + \cos\theta) + \frac{1}{16}\lambda^4 \sin^4\theta.$$

Then

$$E = 2\pi r_0^2 n\left\{2 + \frac{m}{n}\lambda^2(1 - \cos\theta) - \frac{1}{2}\lambda^2 \sin^2\theta + \frac{\lambda^3}{3}(1 - \cos\theta)^2(2 + \cos\theta) + \frac{1}{8}\lambda^4 \sin^4\theta\right\}. \quad (11)$$

In order to obtain a check against Fenn's special solution for the value of $r_0/g = 4$ we have plotted the surface energy as obtained from equation (11) in terms of

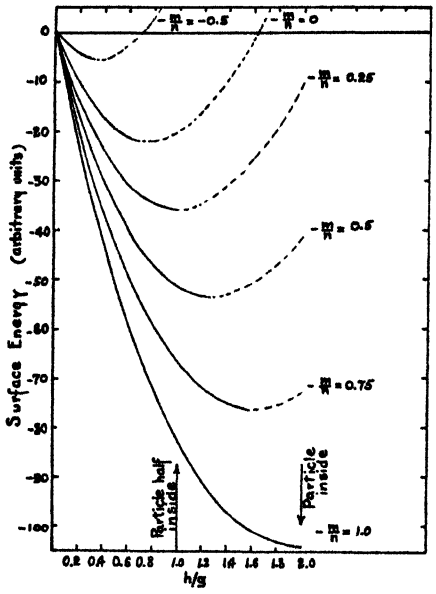
$$(1 - \cos\theta) = \frac{h}{g}.$$

Fig. 3 shows these results. It will be noticed that our curves are to a good degree of precision identical with those obtained from Fenn's (1) solution. As for instance when $-\frac{m}{n} = 0$, the surface energy is a minimum when the particle is immersed in the cell to a depth $h/g = (1 - \cos\theta) = 0.79$, then $\theta = 78°$ and $h = 0.79/4 = 0.20$; while Fenn's data show $y \equiv h = 0.214$ for $-\frac{m}{n} = 0$ at this point of minimum energy.

For values of $-\frac{m}{n} = 0.613$, Fenn finds that the state of minimum surface energy is attained when the particle is immersed to a depth $y \equiv h = 0.375$; while our solution gives $h = 0.357$. A final comparison between the two solutions can be made by comparing the minimum energy values for $-\frac{m}{n} = 0.772$. Fenn obtains $y \equiv h = 0.425$, while our value for h is 0.410.

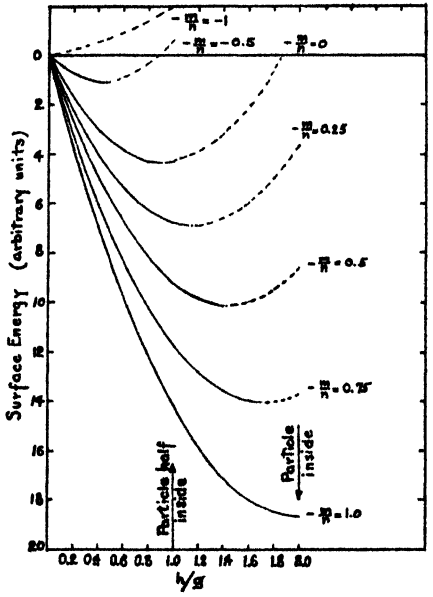
We may conclude that for $\frac{r_0}{g}$ as small as 4 (Fenn's case), our approximate but general solution is quite near the exact solution. It will of course approach more closely to the exact solution the larger $\frac{r_0}{g}$ becomes.

In Fig. 4 are plotted with the aid of our general solution energy values for $r_0/g = 10$, as a function of depth of penetration of the particle into the cell. This case illustrates a common situation met in phagocytosis in which a cell is ten times greater than the particle to be ingested. Similar conditions exist when a particle in the form of a *Staphylococcus aureus* of average diameter from 0.7 to 1.0μ is about to make contact with a large mononuclear lymphocyte with average diameter 12 to 15μ. In this case the solution for $\frac{r_0}{g} = 10$ would describe the physical ingesting situation



$\frac{r_0}{g} = 4$

FIG. 3



$\frac{r_0}{g} = 10$

FIG. 4

These graphs show how the surface energy, expressed in relative units, varies as the particle progressively penetrates the cell, abscissae h/g indicating the depth of penetration of the particle. The particle is half inside the cell for $h/g = 1$, and completely immersed for $h/g = 2$. The increasing values of $-m/n$ indicate the increase in available energy for ingestion due to a change in character of the surface of the particle (or of the cell) to a point ($-m/n = +1$) where the particle may be completely covered by the spreading cell due to the available surface energy alone. Broken lines beyond the minimum indicate that the cell may ingest the particle completely to a point $h/g = 2$ if the cell can obtain the energy to complete the process.

Note that the scale of energy units for $r_0/g = 4$ is five times as great as $r_0/g = 10$.

very closely. Fig. 4 shows how the energy of the expanding cell, as it progressively ingests the particle, passes in every case through a minimum at some specific depth of penetration. This depth of penetration is expressed on the abscissa in terms of h/g. Note that the particle is free for $-\frac{m}{n} = +1$ and completely surrounded by the cell at equilibrium when $-\frac{m}{n} = -1$. These two extreme situations are common to all cases of ingestion and are determined exclusively by the value of the contact angle.

Intermediate values of $-\frac{m}{n}$ are of scientific importance. They show that the energy can be a minimum and in equilibrium at any partial state of ingestion depending on the surface energy of the system, further ingestion being impossible unless the cell draws on some other source of energy, when it could complete the ingestion along the broken curve.

Table I shows the progressive decrease in surface energy with changes in magnitude of $\frac{m}{n}$ for $r_0/g = 10$, at positions of stable equilibrium. Interpolation of the data shows that the particle can be in equilibrium, though

TABLE I

Calculated Values of Minimum Surface Energy, and Depth of Penetration of a Particle for Various Values of $-\frac{m}{n}$, *when* $\frac{r_0}{g} = 10$

$-\frac{m}{n}$	$A°$	Minimum energy arbitrary units	Depth of penetration $\frac{h}{g}$	
+1.00	0	−18.67	2.00	Completely in
+0.75	41.5	−14.02	1.70	
+0.50	60	−10.15	1.43	
+0.25	75.5	−6.92	1.15	
+0.10	84	−5.32	1.00	Half in
0.00	90	−4.37	0.90	
−0.50	150	−1.07	0.43	
−1.00	180	0.00	0.00	

Note that only about one-third of the energy is used to submerge the first half of the volume of the particle. A is the contact angle in circular degrees.

only half ingested when $-\frac{m}{n} = 0.10$. The contact angle between particle and cell ($\cos A = 0.10$) is then $A = 84°$. It is of special significance to note that the contact angle is less than 90° when the particle is half submerged. During the remainder of the ingestion the contact angle must swing from this equilibrium position to 180° for complete submergence. A similar situation is encountered when cell to particle diameter is four to one, where at half immersion this contact angle is 81°. For greater ratios of cell to particle diameter this contact angle at half immersion is progressively larger, becoming 90° for contact between a plane surface and half submerged particle.

It is possible for a cell completely to ingest a series of small particles

although it would only partly ingest a large particle of volume equal to the sum of the volumes of the small particles. For example assume the existence of four particles of volumes v_1, v_2, v_3, and v_4 having radii r_1, r_2, r_3, and r_4. Their total volume is $v_1 + v_2 + v_3 + v_4 = V$. The surface energy of this large spherical volume V is $E = 4\pi R^2 T$ while the surface energy of the four small spherical volumes, for simplicity assumed to have equal radii, is $e = 4(4\pi r^2)T$. Since the four small volumes shall equal the large volume, it follows that the radius R is $(4)^{1/3}$ or 1.587 times as large as r. Hence the surface energy available from the ingestion of the four small volumes if ingested successively is $e = 4\pi(4)T$, while the single sphere of equal volume only has surface energy $E = 4\pi(2.52)T$. Apparently then if the single large particle is just able to be completely ingested, it is much easier for the cell to ingest a group of small particles, having the same resultant volume as a large single particle, provided they are ingested successively.

In closing we wish to join Fenn (1) in emphasizing Tait's (2) conclusion, borne out by the above analysis, that only unstable cells tend to be phagocytic. When they have reached their stable minimum surface energy state with respect to their environment, no further ingestion can take place unless energy is expended by the cell to complete the ingestion, indicated by the broken line of Figs. 3 and 4.

Through sensitizing the surface of the particle, which implies resurfacing the particle at least with a monomolecular layer with an interfacial surface tension depressant, the magnitude of $-\frac{m}{n}$ is made larger because of the decrease in the magnitude of n. Under these circumstances the particle can sink deeper into the cell before the equilibrium position is attained. Finally, if during ingestion the cell removes this monomolecular layer from the particle, so that the interface PC develops a larger surface tension, ingestion will cease and the particle might be completely ejected because of the cessation of the spreading with subsequent contraction of the cell material.

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2. Tait, J., *Quart. J. Exp. Physiol.*, 1918–20, **12**, 1.

TEMPERATURE AND CRITICAL ILLUMINATION FOR REACTION TO FLICKERING LIGHT

VI. Flash Duration Varied

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I

For animals with simplex, structurally uncomplicated, visual systems the dependence of critical intensity I upon flash frequency F in reaction to visual flicker is described by a single symmetrical probability integral in log I.[1] Vertebrates with duplex visual systems exhibit two such segments, overlapping to various degrees. The parameters of the curve are known to be modifiable as functions of the form of the light-dark succession in the flash cycle, and the proportion of light time to dark time,[2] the retinal area excited,[3] the temperature,[4] and the wave-length composition of the light,[5] and the state of visual adaptation.[6] The theory of the origin of this non-specific type of performance contour has been derived from the considerations that the magnitude of sensory effect (E) produced by the interrupted light at the point of response is proportional to F[7] and is measured by the integral of a frequency distribution of $-I \cdot d(1/I)$ and consequently of $d \log I$.[8] Sensory elements of excitability $1/I$ contribute to sensory effect E

[1] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1938–39, *J. Gen. Physiol*, **22**, 311, 451; Crozier, W. J., and Wolf, E., 1938–39, *J. Gen. Physiol.*, **22**, 555.

[2] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1937–38, *J. Gen. Physiol.*, **21**, 313, 463.

[3] *Cf.* discussion and data in Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1937–38, *J. Gen. Physiol.*, **21**, 223.

[4] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936–37, *J. Gen. Physiol.*, **20**, 393, 411; 1938–39, **22**, 311; 1938, *Proc. Nat. Acad. Sc.*, **24**, 216; Crozier, W. J., 1939, *Proc. Nat. Acad. Sc.*, **25**, 78; Crozier, W. J., and Wolf, E., 1938–39, *J. Gen. Physiol.*, **22**, 487, 795; 1939–40, **23**, 149; *Proc. Nat. Acad. Sc.*, **25**, 171, 176; Crozier, W. J., 1940, *Proc. Symp. on Temp.*, Inst. Phys., New York, in press.

[5] *Cf.* Hecht, S., and Smith, E. L., 1935–36, *J. Gen. Physiol.*, **19**, 979.

[6] *Cf.* Crozier, W. J., and Wolf, E., 1939, *Biol. Bull.*, **77**, 126.

[7] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1935–36, *J. Gen. Physiol.*, **20**, 411; 1937–38, **21**, 17.

[8] Crozier, W. J., 1940, The theory of the visual threshold, in preparation.

over the finite time involved in the determination of response with a magnitude which declines with the intensity.

The effects of altering (1) the percentage light time $[100\ t_L/(t_L + t_D)]$ in the flash cycle and (2) the temperature should give a direct test of this conception. An increase of dark time permits each flash to act on a larger number of non-refractory elements, because of greater chance of recovery during a longer dark time. Hence the total number of units of effect obtainable ($\equiv F_{max.}$) should be greater, while a given level of effect is secured with a lower intensity. But since the neural elements concerned are the same, and their basic excitabilities, $\sigma'_{\log I}$ (the standard deviation of the frequency distribution of effects produced, with $F_{max.}$ = 100 per cent) should not be modified. These are the findings;[2] $F_{max.}$ and the abscissa of the inflection (τ') of F-log I are each rectilinear functions of the percentage dark time in the flash cycle, but $\sigma'_{\log I}$ is invariant. On the other hand, increasing the temperature merely increases the metabolic reaction velocities responsible for the capacity to be excited, $1/I$; hence $F_{max.}$ and $\sigma'_{\log I}$ are not functions of temperature but τ' is.[4] These effects have been secured in animals of such morphological diversity that their origin and basis in the dynamical statistics of cellular assemblages, rather than in any other way, seem obvious and assured. The analytical form of the F-log I function is non-specific; its parameters are specific and determinate, since they are fixed by the genetic make-up of the animal[9] and their magnitudes exhibit rational dependence on variables which may be used to control them.[2, 4]

One important point remains to be examined. The influence of temperature on τ' has been studied[4] with $t_L = t_D$. The rôle of the light-time ratio has been studied[2] with the temperature constant at 21.5°. If the kind of interpretation previously suggested is correct, it is required that the exact mode of dependence of τ' on the temperature must be the same with $t_L = 0.1$ as with $t_L = 0.9$, and the $F_{max.}$ and $\sigma'_{\log I}$ must be independent of temperature at all values of the light-time fraction.[9a]

[9] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1937, *Proc. Nat. Acad. Sc.*, **23**, 516; 1937–38, *J. Gen. Physiol.*, **21**, 17; Crozier, W. J., and Wolf, E., 1938, *Proc. Nat. Acad. Sc.*, **24**, 542; 1938–39, *J. Gen. Physiol.*, **22**, 463.

[9a] Cases do arise, however, in which the number of potentially excitable elements is a function of temperature. Such an instance is found, for example, in the relation of exposure time to current strength for production of a given size of action current in nerve (*cf.* Crozier, W. J., 1937, *Proc. Nat. Acad. Sc.*, **23**, 71); presumably the same situation can be presented by the time intensity relation for photic excitation, under certain conditions. In the case of nerve the law of the temperature effect on excitability can be

The requirements are exceptionally rigorous. The outcome of the tests is a definite verification of the several expectations mentioned. A further instance is thus provided of the analytical separation of the properties of metabolically determined $1/I$ and of the purely statistical (distribution) factors producing the form of the F-log I contour; μ for $1/I$ has already been shown to be independent of the form of the curve.[4] It is also of significance for the general theory of temperature dependence and of the origin of the invariance of temperature characteristics in the performance of biological systems.

II

For these tests the responses of young turtles, *Pseudemys scripta, ssp.*, are especially advantageous.[10] The F-log I curve is simplex; the relation of $1/I$ to temperature (with $t_L = t_D$) exhibits a critical temperature at 29.5°, with a quite high μ from 12 to 30° (μ = 26,700) and a much lower one (12,400) above 30°; the responses are sharp and precise, and individual differences in a lot of individuals provide internal evidence of the precision of the observations.[10] The nature of the temperature function is such as to give a real chance to detect even slight alterations which might be due to change of t_L/t_D were these to occur with this animal. Below 30° the μ plot is steep, and the presence of the "break" at 29.5° indicates that two distinct processes are involved in the control of $1/I$.

The procedure used and the methods of calculation have been described.[11, 12] A group of 10 numbered individuals (3.24 to 3.66 cm. carapace length) was taken from a larger number acclimated to laboratory conditions. These same 10 were used throughout the present experiments. At each temperature and light-time fraction three observations were made with each dark-adapted individual at each flash frequency used. The means (I_1) of the three readings of I for the 10 individuals are averaged and entered as I_m in Tables I and II. The P.E. of the dispersion of the individual

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obtained from the behavior of the value of log t $_{\text{infln.}}$ for the probability integral relating $1/c$ to log time, that is of the chronaxie τ, provided $\sigma'_{\log t}$ remains constant—as it is observed to do. Otherwise, the Arrhenius plot for log $1/t$ *vs.* $1/T_{\text{abs.}}$ cannot be rectilinear if t is the time for excitation by a fixed magnitude of current.

[10] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1938, *Proc. Nat. Acad. Sc.*, **24**, 125, 216; 1938–39, *J. Gen. Physiol.*, **22**, 311.

[11] Wolf, E., and Zerrahn-Wolf, G., 1935–36, *J. Gen. Physiol.*, **19**, 495; Crozier, W. J., 1935–36, *J. Gen. Physiol.*, **19**, 503.

[12] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936–37, *J. Gen. Physiol.*, **20**, 211, 393; 1937–38, **21**, 313, 363.

means is also given. Certain properties of this variation are discussed subsequently.

The methods used to control temperature[4, 10] and light-time fraction[2] have been described. The temperatures employed were arranged in random order. Check observations assured that even at 36° and at 12° as extreme adaptation temperatures the effects were freely reversible. The behavior of the turtles in providing the response signifying the establishment of the critical intensity has also been discussed;[10] it is to be added merely that with decreasing t_L, as with rising temperature, the vigor and amplitude of the responses is found to increase.[12] This has no effect on the variability of critical intensity (*cf.* Figs. 8, 9).

In all of these experiments we have found it necessary and important to begin each set of observations with dark-adapted animals. According to Birukow[13] this is not required for the turtles *Emys* and *Testudo*. We have pointed out that, for crayfish and frog, light adaptation reduces the F range and increases the critical intensities,[14] and that this produces a profound contrast in the F-log I curves gotten for frogs by Birukow[15] and by our procedure. For man it is well known that the critical flash frequency is a very definite (and superficially complex) function of the level of photic adaptation.[16] The point is easily demonstrated qualitatively for *Pseudemys*. *Pseudemys* were adapted for 30 minutes under light of *ca.* 6,550 ml. on a white background and surrounded by white walls. They were then tested for response at $F = 20$. At this temperature (21.5°), log I_m for the critical flash intensity = $\bar{2}.204$ when the animals are previously dark-adapted. When exposed for light adaptation the head was always extended and the eyelids open, so that full adaptation was assured. On transfer to the apparatus for testing flicker response the turtles are restless, but good responses by the standard procedure can be observed after 10 to 20 seconds. Successive readings of the critical intensity get gradually smaller as the effect of light adaptation wears off. Data on 4 individuals, giving successive measurements (log I) up to 2 minutes after removal to the apparatus, illustrate this:

Animal No.	1	2	3	4
	$\bar{2}.90$	$\bar{2}.86$	$\bar{1}.05$	$\bar{1}.08$
	$\bar{2}.87$	$\bar{2}.70$	$\bar{2}.85$	$\bar{1}.02$
	$\bar{2}.75$	$\bar{2}.68$	$\bar{2}.72$	$\bar{2}.86$
	$\bar{2}.83$	$\bar{2}.65$	$\bar{2}.56$	$\bar{2}.78$
	$\bar{2}.65$	$\bar{2}.40$	$\bar{2}.38$	$\bar{2}.67$
	$\bar{2}.45$		$\bar{2}.34$	

[13] Birukow, G., 1939, *Z. vergleich. Physiol.*, **27**, 322.

[14] Crozier, W. J., and Wolf, E., 1939, *Biol. Bull.*, **77**, 126; 1939–40, *J. Gen. Physiol.*, **23**, 229.

[15] Birukow, G., 1937, *Z. vergleich. Physiol.*, **25**, 92.

[16] Lythgoe, R. J., and Tansley, K., 1929, *Great Britain Med. Research Council, Special Rep. Series, No. 134.*

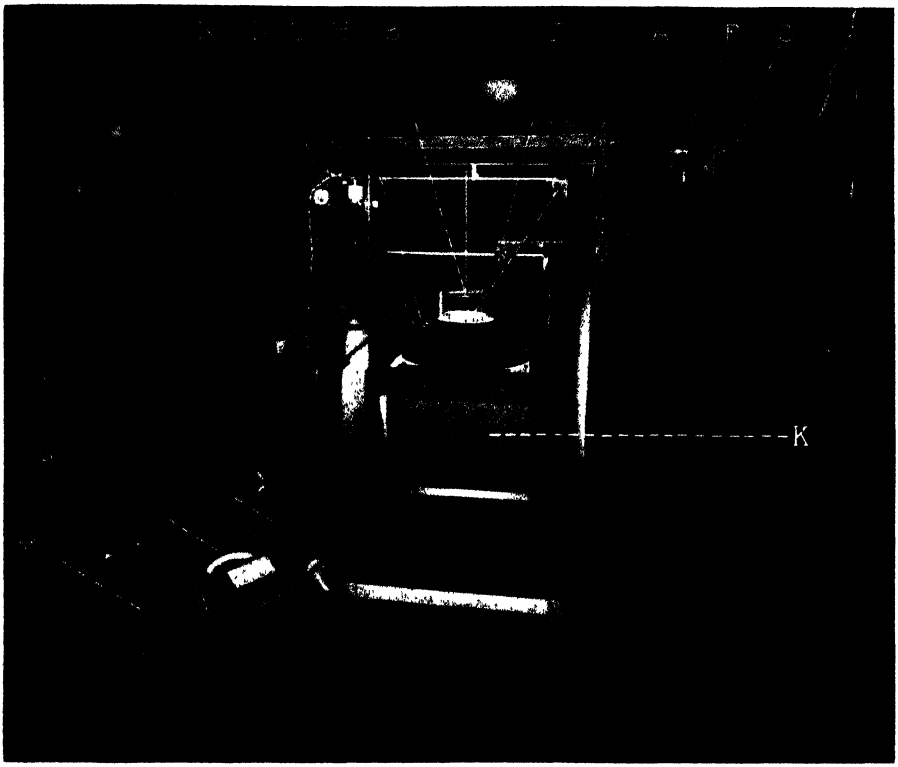

FIG. 1. The apparatus producing flicker by rotation of a striped cylinder A, a cylinder (inside diameter = 14.5 cm.), the one shown has 40 opaque stripes. The animal under observation is in a cylindrical dish of thin glass, within the cylinder. The cylinder in its holder B is driven through a gear system by the variable speed motor C, coarse and fine rheostats D and E adjust its speed. The precision magneto F is geared to the same shaft, the voltage it develops, read on the millivoltmeter G, gives the rotation frequency of the cylinder in terms of calibration charts for the different gear systems used. Light from a lamp on an optical bench is admitted through the square diaphragm at H, controlled at I, a vernier scale (illuminated by use of a foot switch) can be read in the telescope J to give the diaphragm-opening, and thus by way of calibration charts the intensity (within the cylinder) due to a flash. The light is reflected vertically by the plate mirror K and is then horizontally diffused by the oxide coated cone L. The bench M is for recording and monitoring the cylinder speed. During observations the curtain N excludes light from red pilot lamps, there being no other illumination in the dark room. (Thermostat tanks for dark adaptation are immediately at the right.)

The effect of proper preliminary dark adaptation in stabilizing the critical intensity, and the direction of the shift under light adaptation, are therefore the same with *Pseudemys* as with other animals.

It is worth recording that on one occasion two successive sets of observations were found to be discordant by a small amount, about 0.1 log unit; this was traced to the previously unnoticed drift of the thermostat temperature owing to the "sticking" of the regulator, the amount being 0.3°. In another case a slight error in the construction of a particular striped cylinder was found to be responsible for a persistent 0.2 log unit shift in the value of I_m for $t_L/t_D = 9/1$ at $F = 10$. Unconscious experiments of this sort illustrate the kind of precision possible in these experiments if rigorous attention is given to their details.

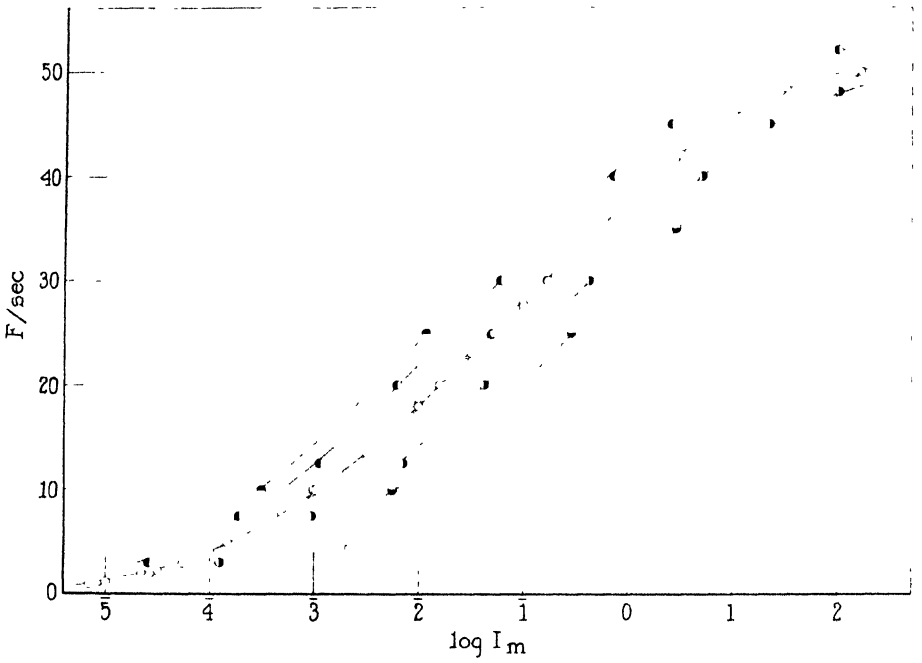

FIG. 2. Log mean critical intensity I_m as a function of flash frequency F, at 21.5°, for several proportions of light-time (t_L) to dark-time (t_D) in the flash cycle $t_L = 0.10$, 0.25, 0.50, 0.75, and $0.50 \times t_L + t_D$; *i.e.*, $(t_L + t_D) = 1/F$. See Table I. Data for $t_L = 0.50\ t_D$ are from a previous paper;[10] here the circlets with tags refer to measurements of F_m; several determinations at $t_L = 0.50$ with the animals of Tables I and II are included to show reproducibility. The curves are adjusted probability integrals (*cf.* Fig. 3)

For technical reasons[2] it is difficult to obtain light-time ratios greater than 9:1 or less than 1:9, and with these the complete response contours cannot be measured. These limitations arise because of the difficulty of preparing striped cylinders[12] with the necessary accuracy and because flash intensities above antilog 2.3 millilamberts cannot be controlled with precision in the apparatus (*cf.* Fig. 1). Since, however, the form of the F-log I curve does not change with temperature we are able to use $t_L = 0.1$

and 0.9 at any fixed flash frequency F (below $F = 35$) and thus obtain data at cylinder rotation speeds which are favorable for precise determinations at various temperatures. On the other hand the F-log I function can be gotten over essentially its whole range at 21.5° with $t_L = 0.25$ and 0.75. We shall consider these measurements first, since it is desirable to establish the generality of the rules already found for this matter with *Anax* (dragonfly nymph) and *Enneacanthus* (sunfish).[2]

TABLE I

Critical flash intensities (as log mean I, millilamberts) with P.E.$_1$, for response of *Pseudemys* at 21.5°, for various flash frequencies, with the proportion of light-time (t_L) in the flash cycle at 10, 25, 75, and 90 per cent; values of F_m at log I = 2.000. See Figs. 2 and 3.

F/sec.	t_L = 10 *per cent*		25 *per cent*		75 *per cent*		90 *per cent*	
	log I_m	log P.E.$_1$	log I_m	log P.E.$_1$	log I_m	log P.E.$_1$	log I_m	log P.E.$_1$
3			$\bar{5}.4198$	$\bar{6}.0414$	$\bar{4}.0962$	$\bar{6}.5679$		
7.5			$\bar{4}.2794$	$\bar{6}.9108$	$\bar{4}.9804$	$\bar{5}.5268$		
10	$\bar{4}.4954$	$\bar{5}.1471$					$\bar{3}.7527$	$\bar{4}.1467$
12.5			$\bar{3}.0596$	$\bar{5}.3444$	$\bar{3}.8526$	$\bar{4}.3923$		
20			$\bar{3}.7923$	$\bar{4}.0906$	$\bar{2}.6227$	$\bar{3}.0878$		
25	$\bar{2}.0719$	$\bar{4}.6549$					$\bar{1}.4597$	$\bar{3}.8392$
30			$\bar{2}.7808$	$\bar{3}.1520$	$\bar{1}.6380$	$\bar{2}.1746$		
35							0.4677	$\bar{2}.8475$
40			$\bar{1}.8541$	$\bar{2}.3375$	0.7072	$\bar{1}.3392$		
45			0.4140	$\bar{1}.1126$	1.3452	$\bar{1}.9239$		
52.06	±0.614		2.000					
48.09	±0.460				2.000			

III

Table I contains the measurements from which the properties of the *Pseudemys* F-log I contour in terms of the light-time fraction at constant temperature may be deduced. It is apparent from Fig. 2 that, as with *Anax* and *Enneacanthus*[2] the abscissa of inflection (τ') is decreased and the maximum F increased when the percentage light-time in the flash cycle is reduced. It is also clear, as in the previous cases, that $\sigma'_{\log I}$ is not affected (Fig. 3). Fig. 6 shows that, again as before,[2] $F_{\max.}$ and τ' are related rectilinearly when t_L is changed. (For the abbreviated series with $t_L = 0.1$ and 0.9, $F_{\max.}$ was found with the help of Fig. 4, and from this the computed positions of the points in Fig. 3 were found to give rectilinearity with the values of τ' used in Fig. 4 and with the constancy of slope

showing that $\sigma'_{\log I}$ is invariant.) The relations of $F_{max.}$ and τ' to the percentage light-time are shown in Figs. 4 and 5. They are essentially rectilinear, as with *Anax* and *Enneacanthus*.[2] The proportionality constants are not the same as in the other cases, however, indicating that these constants depend on the nature of the visual system in each instance—although the several proportionality constants for τ' *vs.* $100\ t_L/(t_L + t_D)$ do not differ very greatly.

IV

With $t_L = t_D$ it was found that log $(1/I)$ at fixed F obeys the Arrhenius equation as a function of temperature.[4] For certain fishes the same temperature characteristic μ obtains over the entire usable range of temperatures;[17] for other forms,[18] including *Pseudemys*,[10] an intermediate critical temperature is observed. The calculation of μ is made from the slopes of the lines on plots of log $(1/I)$ *vs.* $1/T_{abs.}$. Only in very rare instances are such plots curvilinear, then requiring further analysis.[19] We have already indicated that if the chemical control of excitability is to demonstrate the operation of governing mechanisms with the properties of simple catalytic reaction velocities, then the slopes of the μ plot for $1/I$ must be independent of the light-time fraction in the flash cycle. The data of Table II, plotted in Fig. 7, show that this requirement is satisfied.

In Fig. 7 log $(1/I_m)$ is given as a function of $1/T_{abs.}$ It is apparent that in the immediate neighborhood of 30°C. there occurs in each series a sharp break in the graph, and that on either side of this point the slopes are identical for all three values of the percentage light-time. The magnitudes of μ computed from the slopes are 12,400 cals. above 30° and 26,500 cals. below 30°. These are identical with the results previously gotten[10] for $t_L = 0.5$ at $F = 20$ and $F = 30$. With respect to t_L as with respect to F, the temperature characteristics are invariant. If this were not the case it would follow either that the conceptions of the two different modes of action of t_L and of temperature on the morphology of the F-log I curve are imperfect or incorrect, or that the theory of the nature of temperature characteristics is inadequate,—if not both.

A special point involves the use for this test of a case in which, as in Fig. 7,

[17] Crozier, W. J., and Wolf, E., 1939, *Proc. Nat. Acad. Sc.*, **25**, 171, 176; 1939–40, *J. Gen. Physiol.*, **23**, 143.

[18] Crozier, W. J., 1939, *Proc. Nat. Acad. Sc.*, **25**, 78; Crozier, W. J., and Wolf, E., 1938–39, *J. Gen. Physiol.*, **22**, 487.

[19] Crozier, W. J., 1939, *Proc. Nat. Acad. Sc.*, **25**, 78; Crozier, W. J., and Wolf, E., 1938–39, *J. Gen. Physiol.*, **22**, 795.

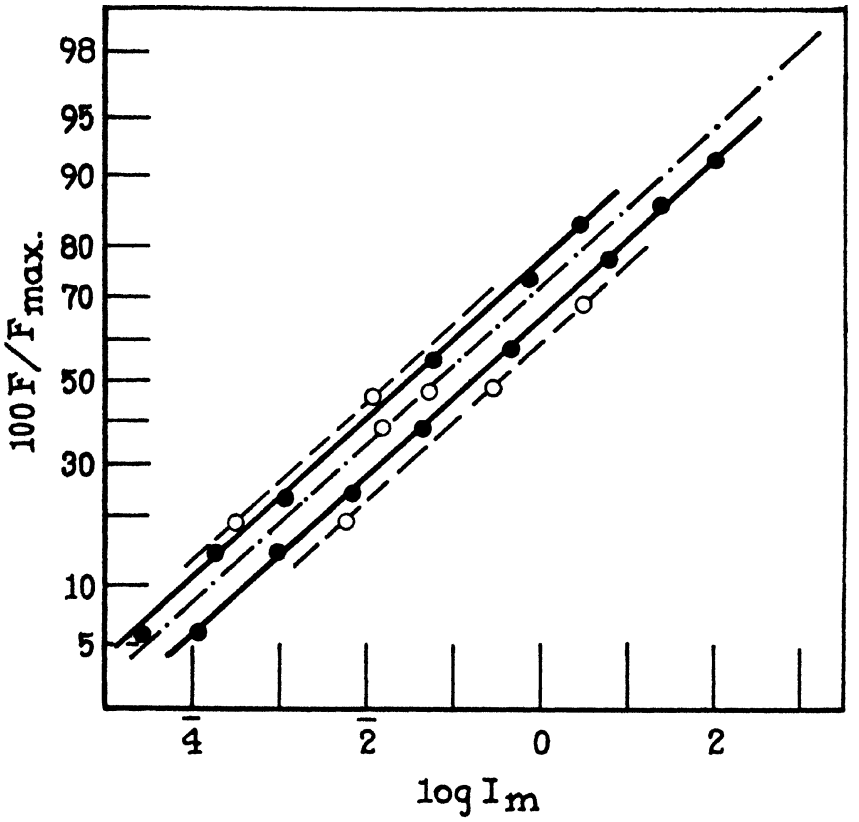

FIG. 3. The data of Fig. 2 on a probability grid, each series computed in terms of its own $F_{max.}$ (*cf.* Figs. 4 and 6). The slopes are drawn equal; $\sigma'_{\log I}$ is consequently constant and independent of the light-time fraction. (For $t_L = 0.5$, the line is taken from earlier work,[10] with the fresh determinations added.)

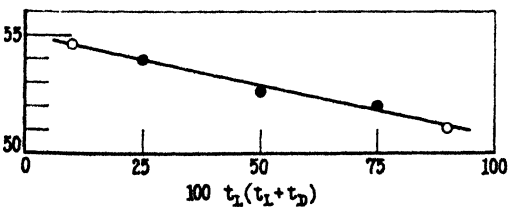

FIG. 4

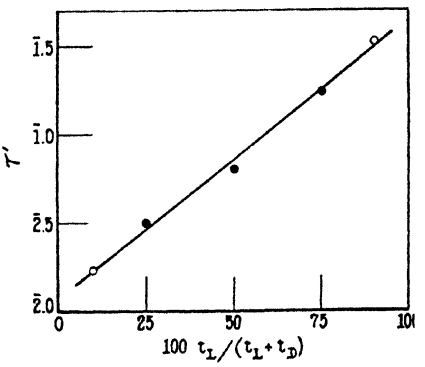

FIG. 5

FIG. 4. $F_{max.}$ decreases rectilinearly as percentage light-time is increased.

FIG. 5. τ' increases rectilinearly as the percentage light-time in the flash cycle is increased.

there occurs a "break" in the Arrhenius plot. Theory here demands[10,18] that there be involved two different, linked, processes which by virtue of some physical change associated with a critical temperature are respectively

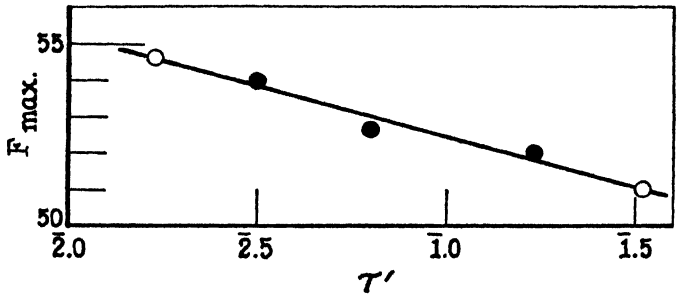

FIG. 6. The asymptotic maximum ordinate ($F_{max.}$) giving adjustment of each series of observations on the probability grid (Fig. 3) declines in rectilinear relation to the increase of the abscissa of the inflection point (τ').

TABLE II

Critical flash intensities, as log I_m (millilamberts), with log P.E.$_1$, for response of *Pseudemys* at flash frequency F = 25/sec., with light-times (t_L) = 10, 50, and 90 per cent of the flash cycle, at various temperatures ($t°_{corr.}$); three observations on each of the same 10 individuals at all points. See Figs. 7 and 8. (Three sets from previous measurements[10] at t_L = 50 are given for comparison.)

$t°_{corr.}$	t_L = 10		50		90 *per cent*	
	log I_m	log P.E.$_1$	log I_m	log P.E.$_1$	log I_m	log P.E.$_1$
12.2	$\bar{2}.6696$	$\bar{3}.0124$	$\bar{1}.3272$	$\bar{3}.8674$	0.0162	$\bar{2}.4038$
15.07	$\bar{2}.5164$	$\bar{3}.0394$	$\bar{1}.1596$	$\bar{3}.7222$	$\bar{1}.8761$	$\bar{2}.2014$
18.03	$\bar{2}.3098$	$\bar{4}.7458$	$\bar{2}.9441$	$\bar{3}.3105$	$\bar{1}.6779$	$\bar{2}.0469$
21.5	$\bar{2}.0719$	$\bar{4}.6549$	$\bar{2}.7090$	$\bar{3}.2499$	$\bar{1}.4597$	$\bar{3}.8392$
			$\bar{2}.7097$	$\bar{3}.3579$		
			$\bar{2}.7016$	$\bar{3}.6671$		
23.95	$\bar{3}.8968$	$\bar{4}.2882$	$\bar{2}.5176$	$\bar{3}.1136$	$\bar{1}.2425$	$\bar{3}.6863$
27.7	$\bar{3}.6221$	$\bar{5}.9802$	$\bar{2}.2753$	$\bar{4}.8584$	$\bar{1}.0237$	$\bar{3}.5594$
29.50	$\bar{3}.5452$	$\bar{4}.0175$	$\bar{2}.1711$	$\bar{4}.5508$	$\bar{2}.9015$	$\bar{3}.2744$
			$\bar{2}.1784$	$\bar{4}.6392$		
31.32	$\bar{3}.4651$	$\bar{4}.0195$	$\bar{2}.1186$	$\bar{4}.7534$	$\bar{2}.8361$	$\bar{3}.2903$
32.80	$\bar{3}.4204$	$\bar{5}.8364$	$\bar{2}.0592$	$\bar{4}.6088$	$\bar{2}.7873$	$\bar{4}.9817$
34.30	$\bar{3}.3936$	$\bar{4}.0622$	$\bar{3}.9836$	$\bar{4}.4241$	$\bar{2}.7366$	$\bar{3}.2358$
35.83	$\bar{3}.3288$	$\bar{5}.9339$	$\bar{3}.9410$	$\bar{4}.4145$	$\bar{2}.6983$	$\bar{3}.1676$

in control on either side of the critical temperature. The reality of this break is amply evident in Fig. 7. It would not be surprising to find that the use of an additional variable, such as the light-time fraction, would shift the intercepts of the two segments of the graph to different extents.

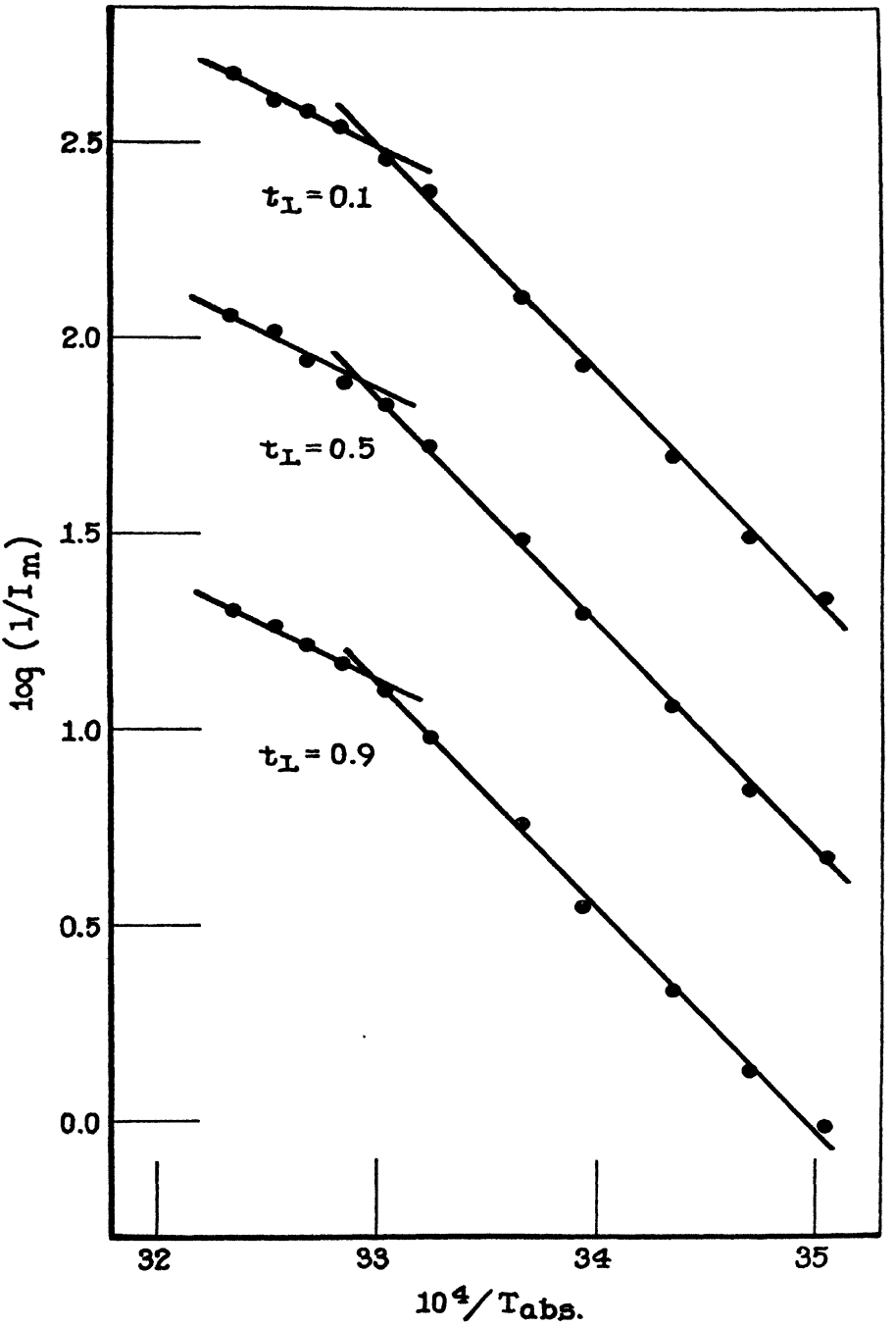

FIG. 7. Log $(1/I_m)$ as a function of $1/T_{abs.}$, for three values of the percentage light-time, at one flash frequency (F = 25/sec.); see Table II. The slopes of the lines on either side of 29.5° + give μ = 12,400 and 26,500 cals. These slopes are the same with

An effect of this kind is quite obvious in the case of the data on the sunfish:[18] the relative positions of the low and high temperature segments are not the same for the "rod" and "cone" portions of the duplex F-log I curve. Other instances are known.[20] This could bring about a slight shift in the critical temperature as t_L is altered, such as is seen in Fig. 7. It could also appear in a difference between different lots of individuals. (While it is not our desire to emphasize in any way the slight differences found in Fig. 7, it is perhaps legitimate to suggest that by the systematic use of additional variables in other cases light might well be thrown on the nature of the phenomena operating at critical temperatures.) Before this can be evaluated, however, it is necessary to consider the variation of I_1 as a function of t_L and of T. We have already shown that slight individual differences tend to persist in *Pseudemys* for longer times than we have noticed with any other animals we have tested.[10]

V

It was shown for the lot of *Pseudemys* previously used[10] that the variation of I_1, measured by P.E._{1I_1}, was rectilinearly related to I_m, and was apparently independent of temperature. In earlier experiments with *Anax* and sunfish it appeared that for any level of I_m the variation of I_1 was slightly greater at an intermediate temperature than at the two extremes.[4] It is not so in the present data, which may be due to improvement of technic or other causes. This can be tested more completely in the present data, with particular reference to the fact that independent evidence may be obtained as to the simplicity or complexity of the processes controlling $1/I$. Simplicity of the controlling process over a given range of temperatures is implied by the constancy of μ and by its invariance with respect to t_L. In a case where complexity of control is required by the inconstancy of μ the variation of I_1 is definitely not a constant percentage of I_m.[19] This supplies an empirical test of the idea that constancy in the relative variation of performance, under changed values of experimentally independent variables affecting the measure of performance, necessarily indicates simplicity of the controlling process, since these independent variables must then be conceived to affect all the elements of the situation in the same proportionate way. The sort of organization making this result possible is to be defined operationally as simple, in virtue of its unitary character.

[20] *Cf.* Crozier, W. J., 1934–35, *J. Gen. Physiol.*, **18**, 801; and experiments with the effects of low temperatures, as yet unpublished.

It also appeared, however, that the raw variation of I_1 was in part influenced by the fact that the individuals of the original lot of 10 tended significantly to maintain their relative excitabilities from day to day. The rôle of this property must be examined in the present case also. We will first consider the gross P.E. figures given in Tables I and II.

Fig. 8 shows that with temperature constant and the percentage light-time varied, the rule of direct proportionality between P.E.$_1$ and I_m is not distorted; the same is now shown in Fig. 9 for the variation of both t_L and temperature. The bands are essentially identical; the proportionality factor k in $I_m = k(\text{P.E.}_{1I_1})$ is the same, and does not differ significantly from that already on record for the first lot of *Pseudemys* with temperature and F as variables.[10] Consequently the homogeneity of the process measured by $1/I$ as an index of excitability, with respect to the significant relations to flash frequency, light-time ratio, temperature, and critical intensity, is objectively confirmed.

When the relative sensitivities in the set of 10 *Pseudemys* are examined it is observed that, as before,[10] these are not distributed at random. Individuals 5, 1, and 9 tend to be much more consistently at one end (5, 9) or the other (1) of the sensitivity rank order than do the others. Thus the mean rank order number of No. 5 in 36 sets of measurements with temperature and t_L/t_D varied is 1.67; of No. 1 it is 8.26. (No. 5 happens to be the smallest individual, carapace length 32.4 mm; but for the others—32.8 to 36.6 mm.—there is no correlation with sensitivity.) With these two and No. 9 excluded, the rank order numbers of the others are practically random. The individual rank order numbers have no relation to the order of use in a series of readings. In the second lot of 22 sets of measurements, with t_L/t_D varied at 21.5°, the mean rank order numbers for No. 5 and No. 1 are respectively 1.44 and 8.12. The differences of these numbers from the random mean 5.5 are 6 to 8 times the P.E. of the differences, and are consistently exhibited. For this lot, also, individuals 9 and 10 persistently retain their relative sensitivities. The others vary at random, so far as can be told within the small span of critical intensities, but Nos. 1 and 9 are consistently the least sensitive while 5 and 10 require the smallest critical intensities. If we take Nos. 1 and 5 for purposes of illustration we find (Fig. 10) that the μ plots for Nos. 1 and 5 are quite parallel, but that the junction of the two sections of the graph is slightly different. It is clear that if a large number of determinations were available for a single individual they would form a band with a bend centering at 29.5° or a little above. This explains why, with a small number of

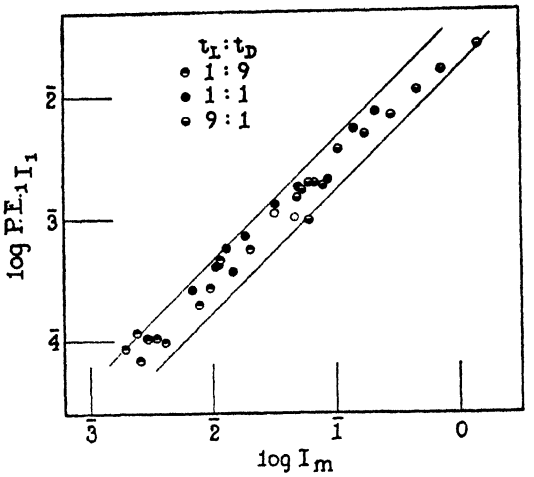

FIG. 8. The constant proportionality of the mean critical intensity and its variation is maintained at constant temperature for different proportions of light-time in the flash cycle. The slope of the band log P.E.$_1$ *vs.* log I_m = 1, and its width is statistically constant.

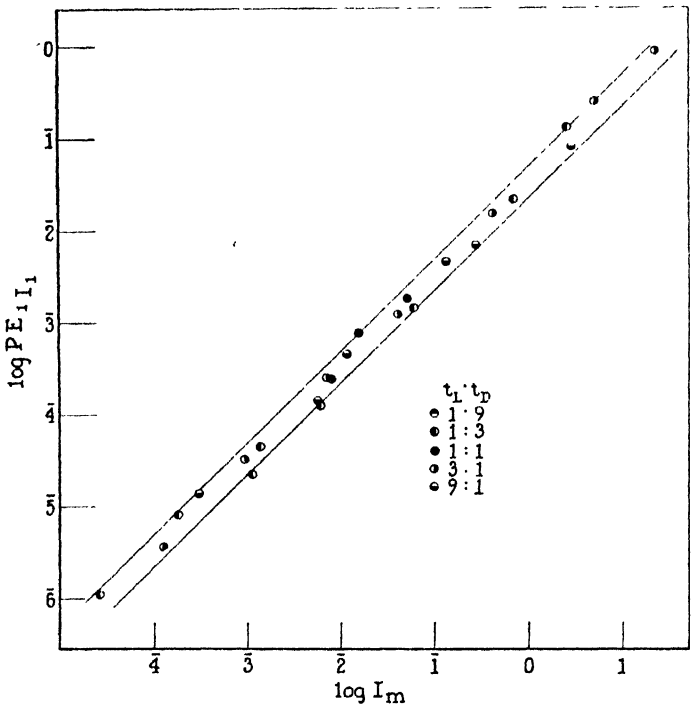

FIG. 9. The constant proportionality of I_m and P.E.$_1$ is unchanged when light-time fraction and temperature are varied; the proportionality constant is the same as in Fig. 8.

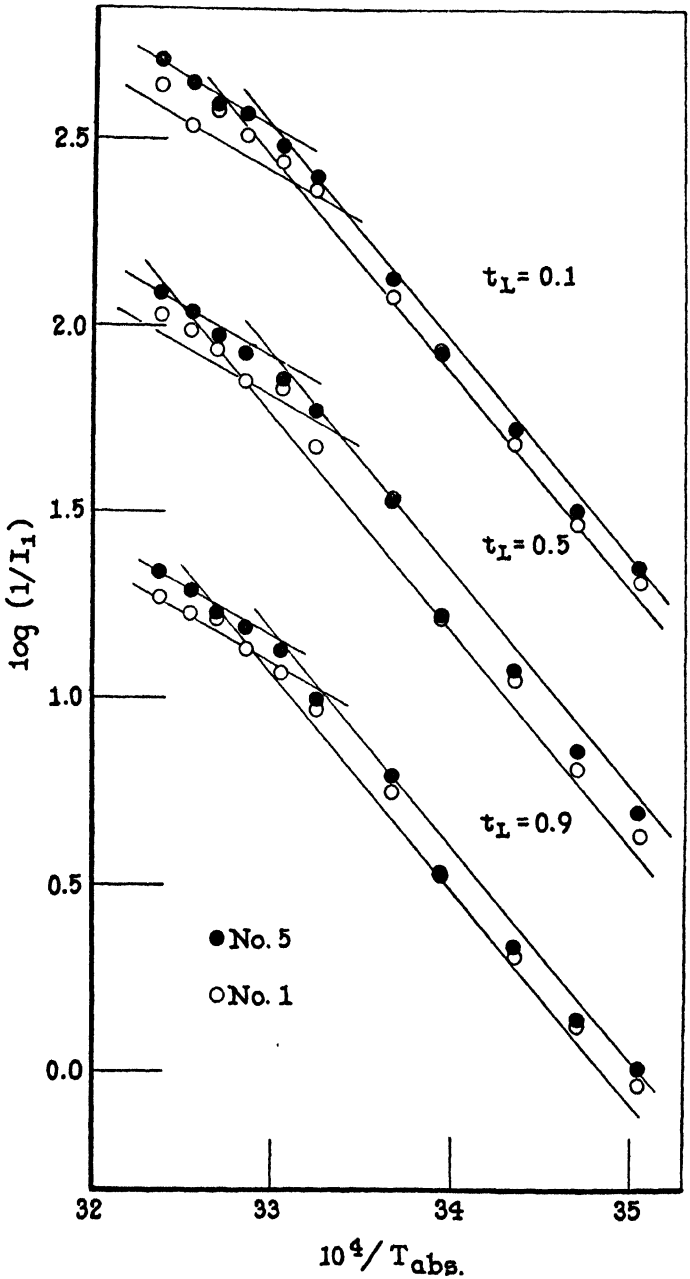

FIG. 10. Log $(1/I_1)$ *vs.* $1/T_{abs.}$ for the two most distinctly different of the 10 individuals from which averages $(1/I_m)$ are used in Fig. 7. See text.

determined points, the region from 28° to 36° is bound to be more uncertain, as in Fig. 7, than elsewhere. A further fact is of course that in the observations made on one day, at one temperature, there is a measure of consistency in the direction of departure of all the readings from the general line of central tendency (*cf.* Fig. 10). This may in part only be due to properties of the apparatus.[10] The greater spread of the points at $t_L = 0.5$, incidentally, is consistent with the data in Fig. 8.

These considerations go some way toward accounting for the systematic departures, slight as they are, seen in the plots of $1/I_m$ (Fig. 7), but it is also to be recognized that the parameters of the F-log I_m function also vary slightly from one animal to another. This has been illustrated before;[10] the same kinds of minor differences appear in the detailed records of the present experiments.

VI

The theory of the meaning of the qualitative relations of the F-log I curve to temperature and to the light-dark ratio respectively has been conceived in the following way.

The temperature controls the velocities of specific catalytic processes which govern excitability; the excitability is measured by $1/I_m$, where I_m is the mean critical intensity for response; the sensory effect produced at this level is proportional to F; consequently, for $dF/d \log I$ constant, $1/I_m$ has the physically appropriate relation to temperature; the shape of F *vs.* log I is not a function of temperature, hence at any F the same temperature characteristic holds for $1/I_m$. This means that in terms of the derivation of the form of $dF/d \log I$ the total number of potential elements of sensory effect involved is independent of the temperature, while the ease with which they can be aroused (in terms of intensity) is dependent on the temperature. In other words, we have to do with a frequency distribution of $-k'I\, d(1/I)$ in which k' is independent of temperature but $1/I$ is not, for a given kind of animal. It is to be remembered that I is the intensity for excitation, or rather that the number of elements in a particular $d \log I$ class gives the appropriate normal occurrence for this value of I—the number of units of effect produced in the interval $(1/I_a + d(1/I)) - (1/I_a)$, where $1/I_a$ is a function of temperature. Consequently $F_{\max.}$ and $\sigma_{\log I}$ are not affected by temperature.

On the other hand, when the light-dark ratio is altered it must be supposed that k' is a function of the dark-time fraction. The frequency with which flashes of a given intensity, at the critical flash frequency F, serve to produce unit sensory effects in elements of excitability $1/I$ is greater

the longer the dark-time fraction. Hence, at given I, F is higher, and $F_{max.}$ greater, in proportion to lengthening of the dark-time fraction. If this effect is one involving k', not $1/I$, then the temperature characteristics for $1/I$ must be the same for all levels of the light-dark ratio. This has been shown to be the case. On any other theoretical basis this kind of result is apparently impossible. It amounts to an analytical separation of the properties of the two factors involved in the production of the frequency distribution of the elemental sensory effects of which the F-log I contour is the integral. The two factors have to do with the excitabilities of the effects, and with the frequencies of their occurrence as a function of intensity. It has been argued that the basic excitabilities form some sort of a frequency distribution of $d(1/I)$, but that over a finite time interval the number of elementary effects gotten by critical intensity I from excitabilities momentarily in the class $[1/I_a + d(1/I)] - [1/I_a]$ will be a declining function of I_a, such that this number $= -k'I$, unless the conditions of excitation are changed. When they are changed by increasing the dark-time fraction the effects are exactly of the kind produced by enlarging the retinal area involved, as already emphasized:[21] $F_{max.}$ is increased, τ' is decreased, but (for a homogeneous area) $\sigma'_{\log I}$ is constant. The reason is that with a longer proportionate dark-time the chance of any flash encountering a potentially excitable unit in a non-refractory state is in this degree increased. The integral of $-k'I \cdot d(1/I)$ is that of $k'd \log I$. Clearly, it is with this k' that purely photochemical (or photoelectric) considerations must be primarily concerned, and curiously enough not with $1/I$. It is of course consistent with this that μ for τ' is constant and independent of the dark-time fraction, and that Talbot's law does not apply to marginal flicker.[2]

Parallel cases are provided by certain other properties of homogeneous cellular populations. Two may be cited briefly. When, with a given interelectrode distance, the strength-duration curve for excitation of a nerve is determined at two temperatures, the curve of $1/C$ *vs.* log t is a probability integral in which the maximum value of $1/C$ is greater at the higher temperature and τ' less, although $\sigma'_{\log t}$ is the same.[22] Increase of temperature makes more of the elementary excitability units available. (In this case enlargement of area by further separation of the electrodes does the same thing for $(1/C)_{max.}$ and τ', but increases $\sigma'_{\log t}$ because of greater variability in the population of units.)

A second case is more obviously pertinent. The photosynthetic activity P of green plant cells, measured by the O_2 liberated in a fixed finite time, gives a log probability

[21] Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1937–38, *J. Gen. Physiol.*, **21**, 223.
[22] Crozier, W. J., 1937, *Proc. Nat. Acad. Sc.*, **23**, 71.

integral in terms of $[CO_2]$. If the intensity of illumination I is increased, $P_{max.}$ increases and τ' (*i.e.* log $[CO_2]$ at the inflection point) is decreased, in direct proportion to I. The higher intensity increases the frequency of work done by each element whose momentary threshold is in terms of $d1/[CO_2]$. On the other hand, when $[CO_2]$ is varied, the P-log I contour merely changes its ordinate scale but τ' is unaltered: the amount of work done by each element is then increased, not its frequency.[23]

The proportionality constant k' has another ascertainable property bearing upon the notion that the effect of the dark-time ratio is to be taken as involving a frequency of excitation. This is most simply seen if we consider log I at the inflection point (that is, our τ'). We have it experimentally that at constant temperature

$$\tau' = -C_1 t_D (t_L + t_D) + C_2 \qquad \text{(Fig. 5).}$$

With t_L/t_D constant we have

$$\tau' = C_3/T + C_4 \qquad \text{(Fig. 7).}$$

We can therefore compare the efficacy of (1) the dark-time fraction and (2) temperature in producing the same kind of shift in τ'. For a given change $\Delta\tau'$ the necessary change in the dark-time percentage is a declining rectilinear function of the change in $1/T$ required to produce the same change in τ'. This is the kind of relationship to be expected if the percentage dark-time is proportional to the logarithm of a *frequency*. This could easily be the result if the dynamic equilibrium in percentage of non-refractory units is the outcome of opposed unlike processes respectively due to excitation and recovery of units in which the intrinsic excitability fluctuates.

The problem here can be phrased in a slightly different form. Suppose that the task had been set to ascertain the temperature characteristics of the percentage light-time required to produce constant performance in the visual reaction system of *Pseudemys*. If the flicker response contour were to be used, a natural reference point would be the inflection of the curve. We could then measure at different temperatures the value of the light-time fraction required to activate 50 per cent of the elements at the same fixed value of log I. The calculation is easily made graphically, or numerically, on the basis that the slope of τ' *vs.* t_L in Fig. 5 is known to be the same at all temperatures. It is obvious that under these conditions the temperature characteristics for t_L $(t_L + t_D)$ at $F = 0.5\ F_{max.}$ must be identical

[23] A more complete account of these questions will appear in another place; *cf.* [22], footnote 5; and [8].

in magnitude with that for $1/I$ at any fixed F and t_L/t_D, since the light-time *ratio* is clearly a logarithmic quantity.

We are grateful to Mrs. E. Wolf for her continued assistance.

SUMMARY

For the turtle *Pseudemys scripta* the temperature characteristics for excitability of the response to visual flicker are found to be independent of flash frequency F, flash intensity I, and proportion of light time in the flash cycle. The maximum F to which the flicker response contour (F *vs.* log I) rises, and the abscissa of its inflection, are rectilinear functions of the percentage light-time in the flash cycle, but μ for $1/I$ at any flash frequency is the same with different values of the percentage light-time (10 to 90 per cent).

These facts, together with the properties of the variation of the critical intensity, objectively demonstrate the essentially simple or unitary character of the controlling events in the mechanism governing the excitability. They also provide a means of further illustrating a procedure whereby certain statistical factors in the performance of a population of units may be analytically separated from excitability properties common to all of the individual units. In particular, it is of general significance that it can be seen how it is possible for biologically exhibited frequencies or rates of performance to provide simple and physically significant relations to temperature, despite the fact that this performance may involve, almost inevitably does involve, the integrated actions of many individual units.

THE CELL SAP OF HYDRODICTYON*

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It is obvious that biological findings are enhanced by comparative studies made on a variety of materials; this has certainly proved to be the case in plants with large multinucleate cells, so much utilized in the last 15 years for problems of permeability, salt accumulation, and bioelectric phenomena. Those most employed have fallen in three orders: Charales, or Charophyta (*Nitella*); Siphonocladiales (*Valonia*); and Siphonales (*Halicystis*). Their comparative study has often revealed common principles, as well as startling differences, which in some cases at least, have been reconciled by experimental treatment. While there are other, somewhat less satisfactory genera in each of these orders (respectively: *Chara*; *Batophora* and *Ernodesmis*; *Bryopsis*), a member of a still different order is even more likely to yield new results, or extend the validity of general principles. The present paper deals with a member of a group almost wholly new for such studies, namely the Chlorococcales; these are generally microscopic algae, but one genus, *Hydrodictyon*, has multinucleate cells reaching considerable size. In *H. reticulatum*, the common water-net, found in ponds the world over, the cells are large enough to be seen by the naked eye, and have been used (under the microscope) for studies of the penetration of alcohol by plasmoptysis or bursting.[1] They are not, however, large enough for individual handling, sap extraction, and bioelectric contacts. Three other species have been described, with larger cells: *H. africanum*, Yamanouchi,[2] with net cells becoming separated into spheres a centimeter in diameter; *H. indicum*, Iyengar;[3] and *H. patenaeforme*, Pocock.[4] The latter, occurring in the same ponds as *H. africanum*, has only recently been described, from South Africa, but it appears to be the

* Aided by grants from The Rockefeller Foundation, and the Research Committee of Stanford University. Collection of material and sap, and some of the chemical and physical measurements by Blinks; detailed chemical analysis by Nielsen.

[1] Holdheide, W., *Planta*, 1931, **15**, 244.

[2] Yamanouchi, S., *Bot. Gaz.*, 1913, **55**, 74.

[3] Iyengar, M. O. P., *J. Indian Bot. Soc.*, 1925, **4**, 315.

[4] Pocock, M. A., *Tr. Roy. Soc. South Africa*, 1937, **24**, 263.

species reported upon here. Smith[5] indicated its occurrence in California several years before Pocock's description, but did not give it a name. It turns out to be identical in almost every respect, however, with *H. patenaeforme*, and will be so designated, lacking sufficient distinction to make it a new species.

Sap analysis of the same species from Africa would be of interest, in comparison with the present form. There is some evidence that *H. africanum* may occur in California, and it may be possible later to report upon it.

Material and Sap Extraction

The Californian *H. patenaeforme* occurs nearly every year in the same spot on the Stanford campus, in a low-lying pasture which is flooded from January to April or May. The young nets appear usually in March as very delicate flat plates or saucers (hence the name), and grow rapidly for 2 months or more, often becoming contorted and twisted into ropes or balls, but sometimes remaining extended in great flat nets up to 20 cm. in diameter, with interstices 2 or 3 cm. wide between the cells. The latter usually join at their ends in threes, although cells at the margin of the net may extend outward alone or in chains. The individual cells become 2 or 3 cm. long, and 1 (rarely 2) mm. in diameter, before assuming the golden, opaque appearance which precedes isogamete formation and collapse of the cell (usually on illumination).

The net is purely and simply aquatic, having no rhizoids or holdfasts like *Nitella*, *Valonia*, and *Halicystis*, which might conceivably influence the absorption of elements from the soil or substrate. In *Hydrodictyon* absorption must be from the surrounding water by the actual cells themselves.

Its very rapid growth might be another advantage for experimental absorption studies, since it grows from an almost invisible fragile net to very large size in about 2 months—an increase of easily 1000 per cent in volume. The season can be extended somewhat by bringing the nets into cooler, shaded pools in the botanical garden, but they rarely survive beyond June in any case.

The cells are readily separated from each other by a slight bend at their junctions, and they then live several weeks when so isolated from the net. This makes them convenient objects for sap extraction free from contaminating material, and for bioelectric contacts at two points on the surface. The constitution of the cell sap is itself of interest, as extending the rather meagre list of organisms from which sap can be obtained in pure state; it also should be known for bioelectric studies, in order to analyze the rôle played by various ions in the maintenance of the normal potential, resistance, and capacity from vacuole to exterior.

The following methods of obtaining sap were employed:

(*a*) For the most careful extraction, the cells were separated and washed in distilled water, then, one at a time, gently drained and dried on filter paper, and placed on a clean glass slide. There the end of the cell was gently pricked with a fine glass needle.

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[5] Smith, G. M., Fresh water algae of the United States, New York, McGraw, Hill, 1933, 487.

Some sap spurted out upon the slide as the cell collapsed; this, and the remainder in the cell was immediately picked up by capillarity or micrometer screw suction into a mounted micropipette, without pressure upon the cells, so that little or no protoplasm or chloroplasts were included in the clear sap. Each cell yielded about 0.01 cc. of sap in this manner, and about 0.5 cc. could be collected in an hour. In all, several cubic centimeters were thus obtained, for samples used to determine the more abundant constituents (K, Cl), as well as for H ion, NH_3, and sulfate tests without the chance of contamination by protoplasm. Several different microtitrations of the chloride were performed on different samples, in order to gain an idea of the variations in Cl at different times, and with the advancing season. It was found to be fairly constant between 0.05 and 0.06 M, older cells generally showing a slightly higher concentration. This pure sap also gave, surprisingly enough, large tests for sulfate, comparable to that in sea water, as borne out by larger scale analyses mentioned below. A charring test was negligible, as was ammonia, by Nessler reagent. Nitrate and phosphate were negligible. There was not enough of sample *a* to analyze for Na, Ca, Mg, or SO_4.

(*b*) To obtain larger samples for analysis of these less abundant elements, but still with a minimum of contamination by outside solution or by protoplasm, groups of smaller cells or complete nets were thoroughly washed in distilled water, drained on filter paper, and then exposed in large crystallizing dishes to a current of warm air until quite dry and flattened to the glass. They were then soaked in several successive small volumes of distilled water to leach out the soluble salts without crushing the cells or releasing any colloidal constituents of the protoplasm, this still being held back by the intact cellulose wall. The volume of this wash water was made up to equal approximately that of the original undried cells, since the cell volume is easily 95 per cent sap. When this was done, the chloride and potassium content, as well as K:Cl ratio, were found to be not very different from that in the directly extracted sap, indicating that the very thin protoplasmic film had not yielded up, or absorbed, appreciable amounts of these elements. This makes it seem likely that the other mineral constituents, chiefly sodium, calcium, and sulfate, are also much the same in such a leached solution as in the pure cell sap; danger of contamination appears the more remote since when protoplasm is definitely included, as in the less pure samples below, these elements are also much the same. On the other hand, magnesium appears in such protoplasmic inclusions, as would be expected from the presence of chlorophyll.

(*c*) Another method of killing the cells, and releasing the sap constituents, was to bring the washed and drained cells to a nearly boiling temperature on a water bath, pouring off the copiously released sap, and leaching again with distilled water to the proper volume, as above. This sap, even from uncrushed cells, was darker in color and tended toward prompter putrefaction than samples *a* and *b*, indicating much greater contamination with protoplasm, organic matter now being released across the cell wall. The inorganic analysis is not very different, however, from that of *a* and *b*.

(*d*) For still cruder extracts, masses of cells, after washing and draining, were crushed with a glass rod, and the copiously extruded sap filtered free of plastids and large protoplasmic particles. Here considerable contamination was inevitable, but the analysis of the ash gave values rather similar to *a*, *b*, and *c*, except for the presence of magnesium and phosphate, obviously derived from the protoplasm.

(*e*) Finally, the residue from this crushing and filtering was saved, and after ashing, was analyzed to give the mineral constituents of the protoplasm and cell wall These

are not notably different from the sap, possibly due to the still strong admixture of the latter with the residue. Strong tests were also obtained here for iron, silica, and phosphate, as might be expected.

Pond Water.—For comparison with the sap, and to indicate the ratio in which the several elements were accumulated in the sap above their external concentration, a large water sample was collected from the shallow pond in which the cells were growing. Since it was very dilute in most of the elements, 5 liters were carefully evaporated down to 500 cc., and the residue, including precipitates, analyzed by the methods employed for the sap. The findings were then divided by 10 to give the original values. The ratios to sap are given in Table II.

Analytical Methods

Owing to the small quantities of sap available, it was necessary to determine as many cations as possible on a single sample. This frequent biological need may justify a

TABLE I

Recoveries from a Known Solution (0.1 Per Cent Glucose Solution Containing Na, K, Ca, and Mg in the Amounts Noted)

	Added	Recovered
	mg.	*mg.*
Na	1.10	0.54; 0.57 (using Cd amalgam)
		1.10; 1.13 (using granular zinc)
K	3.90	3.20; 3.90; 3.80; 3.76; 3.86
Ca	1.00	0.96; 1.04; 1.02; 1.02; 0.99
Mg	0.90	0.87; 0.88; 0.90; 0.98; 0.94

detailed description of methods. A study of the literature[6–9] indicated that it would be possible to adapt some of the recent semi-micro methods to a quantitative procedure for the determination of the desired elements. After the various methods had been fitted together into a procedure such that no reagents containing interfering elements were added, it was tested on known quantities of a 0.1 per cent glucose solution containing all of the elements to be determined. Table I shows the results of these recovery experiments.

It will be noted that the first two recoveries on sodium were poor. Holmes and Kirk[6] in their method for the determination of sodium recommend the reduction of the uranium in the sodium zinc uranyl acetate precipitate by cadmium amalgam. We found that this reagent gave low results (first pair of recoveries), but when granular zinc was substituted for the Cd amalgam a complete reduction was obtained and good recoveries resulted (second pair). Recently Linder and Kirk[10] have published a method

[6] Holmes, B., and Kirk, P. L., *J. Biol. Chem.*, 1936, **116**, 377.

[7] Cruess-Callaghan, G., *Biochem. J.*, London, 1935, **29**, 1081.

[8] Murer, H. K., *Ind. and Eng. Chem., Analytical Edition*, 1937, **9**, 27.

[9] Wilcox, L. V., *Ind. and Eng. Chem., Analytical Edition*, 1937, **9**, 136.

[10] Linder, R., and Kirk, P. L., *Mikrochemie*, 1938, **23**, 269.

for sodium in which is discussed the relative efficiencies of various reducing agents for the uranium, with the conclusion that either granular zinc or cadmium spirals are best suited for the method.

The special reagents employed were the following:

A. For calcium:

1. Saturated $(NH_4)_2C_2O_4$
2. $KMnO_4$ 0.05 N

B. For magnesium:

1. 1 gm. of 8-hydroxyquinoline dissolved in 89 ml. of absolute alcohol. Add 10 ml. concentrated NH_4OH and 1 ml. concentrated HCl. 2 ml. good for 1 mg. Mg.
2. 2.7 gm. $KBrO_3$ and 25 gm. KBr dissolved in 500 ml. H_2O.
3. 6 gm. $Na_2S_2O_3 \cdot 5\ H_2O$ dissolved in 1 liter H_2O. Standardize against KIO_3.

C. For sodium:

1. 10 gm. uranyl acetate $\cdot 2\ H_2O$ and 6 gm. (30 per cent) acetic acid. Make up to 65 gm. with H_2O.
2. 30 gm. $Zn(Ac)_2 \cdot 3\ H_2O$ and 3 gm. (30 per cent) acetic acid. Make up to 65 gm. with H_2O.
3. Warm Nos. 1 and 2 until complete solution is obtained, and then mix together. Allow to stand for 24 hours and filter.
4. 0.02 N ceric sulfate.
5. Phenanthroline ferrous sulfate indicator.
6. Glacial acetic acid saturated with sodium zinc uranyl acetate.

D. For potassium:

1. 0.02 N ceric sulfate
2. Sodium cobaltinitrite
3. 0.02 N ferrous ammonium sulfate.

Procedure

10 gm. of the biological material are placed in a small silica or platinum evaporating dish. One half ml. of concentrated sulfuric acid is added and the material evaporated to dryness on a hot plate or water bath. The dish is then placed in a muffle furnace and ashed at 500-550°C. for about 3 hours or until a white ash is obtained. The ash is taken up with 1 ml. of concentrated HCl. A little water is then added and the dish heated for a few moments on a water bath. The solution is filtered to remove the silica. At this point the solution can be made up to a definite volume, and an aliquot representing 1/10 of the volume set aside for the potassium determination.

Calcium.—Add a few drops of bromine water to the remaining solution to oxidize any ferrous iron present. Boil to remove the bromine and make basic to methyl red with NH_4OH to precipitate the iron and aluminum. The solution is then filtered and washed with hot water. Evaporate the filtrate to about 20 ml.; add 2 ml. of saturated $(NH_4)_2C_2O_4$ to precipitate the calcium; make basic to methyl red and allow to stand overnight. Filter out the CaC_2O_4 through a small, fine-pored, sintered (fritted) glass Gooch crucible and wash several times with dilute ammonia water (4 ml. NH_4OH + 250 ml. H_2O). Save the filtrate for Mg determination. Wash off the bottom of the crucible and dissolve the precipitate with hot 1 N H_2SO_4, drawing it into a test tube placed inside the suction flask. Wash the contents of the tube into a beaker, heat to 90°C., and titrate with 0.05 N $KMnO_4$.[8]

Magnesium.—Evaporate the filtrate from the Ca determination to about 8 ml., and add 5 ml. of 8-hydroxyquinoline reagent. Heat to about 95°C. for a few moments and cool to room temperature. Filter the precipitate, using a Gooch crucible containing a fritted glass filtering disk and wash with 1 N NH_4OH. Transfer the filtrate to a silica evaporating dish and place on a water bath. Dissolve the magnesium precipitate with 4 ml. of 4 N HCl and suck into a 60 ml. test tube. Add 10 ml. of the bromate-bromide solution and immediately stopper the tube. Mix well and let stand for 5 minutes. Add a crystal of KI to reduce the excess bromine and titrate the released I_2 with 0.02 N $Na_2S_2O_3$. Magnesium = equivalents of bromine used/8.[7]

Sodium.—When the filtrate from the magnesium determination has evaporated to dryness it is placed in a muffle furnace and heated to 500°C. long enough to drive off the ammonium salts and the magnesium reagent. Cool and add 1 ml. of 1 N H_2SO_4 and transfer to a 50 ml. beaker. Evaporate to 1 ml. and add 40 ml. of cold, filtered zinc uranyl acetate reagent and let it stand for an hour. Filter through the previously mentioned glass Gooch crucible and wash with glacial acetic acid saturated with sodium zinc uranyl acetate. Dissolve the precipitate with 30 ml. of 2 N H_2SO_4 and suck into a test tube. Pour the solution into a 250 ml. flask and add 2 gm. of granular zinc. Heat to around 80°C. for a few moments to reduce the uranium from valence 6 to 3 and transfer to a beaker. Aerate the solution for a few moments, and oxidize the uranium to a valence of 6 with 0.01 N ceric sulfate.

Potassium.—This can be worked into the above series whenever there is time. Take the aliquot set aside for this determination and make up to 10 ml. Add 1 ml. of 1 N HNO_3. Weigh out roughly 1 gm. of sodium cobaltinitrite for each sample to be precipitated and dissolve in water to make a 20 per cent solution. (Do not make up an excess of the reagent as it does not keep well.) Add slowly, with stirring, 5 ml. of the reagent to the unknown potassium solution. Allow to stand for 2 hours at room temperature. Filter the solution through a Gooch crucible and wash with 0.01 N HNO_3 at least five times. Wash off the bottom of the crucible and place in a beaker containing an excess of 0.02 N ceric sulfate solution and 5 ml. of 1–1 H_2SO_4. Heat on a water bath until the precipitate is dissolved; allow to cool and titrate the excess ceric sulfate with 0.02 N ferrous ammonium sulfate. We have checked the factor 7.1084, given by Wilcox,[9] many times and found it to be correct.

Phosphate was determined by the Zinzadze method.[11] A wet ashing with nitric and sulfuric acids was found satisfactory for decomposing the sample. Losses of phosphate as high as 30 per cent were observed when the material was ashed in a muffle furnace with sulfuric acid as an ash-aid.

Chloride was determined by the conventional $AgNO_3$ titration, using potassium chromate as an indicator.

Sulfate was determined gravimetrically, by precipitation with barium chloride.

DISCUSSION

The results of the analyses are summarized in Table II.

It is clear that potassium strongly predominates, being fifteen to twenty times as concentrated as Na in the sap, although the ratio is markedly reversed in the pond water. Thus the actual accumulation ratio is tre-

[11] Zinzadze, C. H., *Ind. and Eng. Chem., Analytical Edition*, 1935, 7, 227.

mendous, potassium being 4000 times as concentrated in the sap as in the pond water, at the time the analysis was made. It may be that some of this potassium was picked up earlier in the season, as released from other filamentous algae, which appear first in the pools, then die away before the *Hydrodictyon* begins to grow. However, it is at least retained against such a high gradient. That it exists as ionized salt in the sap is shown by the high electrical conductivity, as well as the effective osmotic pressure of the

TABLE II

Composition of Various Samples of Cell Sap and Protoplasm from Hydrodictyon patenaeforme Pocock. Expressed in Moles Per Liter

Sample	K	Na	Ca	Mg	Cl	SO_4	HCO_3, etc.
Pure sap, *a*, 1	0.08				0.059		
Pure sap, *a*, 2	0.101				0.063		
Dried and leached, *b*	0.069	0.0047	0.0008	—	0.0523	0.0086	0.0043
Heated, leached, *c*	0.075	0.0039	0.0017	—	0.052		
Crushed, filtered, *d*	0.062	0.0038	0.0012	—	0.048		
Crushed, filtered, *d*, 2 (1937)	0.0722	0.0045	0.0036	—	0.0532	(0.0056 phosphate)	
Crushed, (1937) *d*, 3	0.075	0.003	0.002	0.0015	0.06	0.0078	0.0067
Cell residue from last, *e*	0.0818	0.0025	0.0023	0.0025	0.058		
Average sap *S* (except *e*)	0.0763	0.004	0.0018	—	0.0553	0.0082	0.0055
Pond water *P*	0.000019	0.0013	0.00108	0.0009	0.00108	0.00077	
Accumulation ratio: (*S*/*P*)	4000	3	1.65	—	51	10.7	

Specific conductivity (several samples, type *d*): 0.009 to 0.0096 (equivalent to 0.077 to 0.083 M KCl)

Tests on fresh *a* samples (purest sap):

pH, 5.5 to 6.0 (by glass electrode, Beckman capillary type)

NH_3 less than 0.001 M (Nessler test)

SO_4 roughly comparable to sea water (actually less by analysis)

Heat test: no charring, indicating little or no organic matter in sap

sap (by plasmolysis, mentioned below). The potassium is also consistently higher than the chloride, by about 0.02 M. This discrepancy is partly made up by the very high sulfate value, about 0.008 M or 0.016 N, nearly 30 per cent of the Cl in equivalents per liter. There is no question about this sulfate being high, since the purest sap, from a single cell without contamination from protoplasm gives a strong test with acid $BaCl_2$, roughly comparable to that in sea water. Even this does not quite balance anions against cations however, so a further search was made. Phosphate and nitrate being negligible in the pure sap, and organic acids probably missing

because of the absence of charring on heating, there remained the possibility of carbonate (bicarbonate at pH 5.5 to 6.0 in the fresh sap). On titrating with HCl to a point acid to methyl red, a figure of 0.0043 N was found for excess base in one case (dried and leached sample); and 0.0067 N in another (crushed sample). Assuming this to be bicarbonate, the cations nearly balance the anions, as shown in Table III.

That there are probably no other major constituents in the cell sap is shown by plasmolysis experiments. Intact cells of *Hydrodictyon* were placed in various dilutions of sea water (as representing a well balanced solution). The cells in sea water/8 and greater dilutions stayed turgid and lived well for several days. Those in sea water/4 showed prompt,

TABLE III

Cell Saps, Expressed As Normality (Equivalents per Liter)

	Hydrodictyon, dried and leached, *b*	*Hydrodictyon*, crushed, *d*	*Nitella clavata*[12, 13]
K	0.0690	0.075	0.0543
Na	0.0047	0.003	0.0100
Ca	0.0016	0.004	0.0204
Mg	—	0.003	0.0354
Cations	0.0753	0.085	0.1201
Cl	0.0532	0.06	0.0908
SO_4	0.0172	0.0156	0.0166
HCO_3	0.0043	0.0067	0.0036 (H_3PO_4)
Anions	0.0747	0.0823	0.1110

continued plasmolysis, while those in sea water/6 were just perceptibly softened, indicating that sea water/7 was close to an isotonic solution. Since this corresponds to about an 0.08 N NaCl solution, which approximates the total normality of the analyzed salts in the sap, it is evident that these must account for practically all the osmotic pressure in the normal cell sap.

Finally, for comparison with the only other fresh water California plant which has had comparable cell sap analysis (and one of the few anywhere in the world), the figures of Hoagland and Davis for *Nitella clavata*[12, 13] are given in Table III.[14] While this *Nitella* grew in a more concentrated pond

[12] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, **5**, 629.

[13] Zscheile, F. P., Jr., *Protoplasma*, 1930, **11**, 481 (revision of Hoagland and Davis).

[14] For comparison of saps from several fresh water and brackish Charophyta of Finland, see Collander, R., *Protoplasma*, 1936, **25**, 201.

water than the *Hydrodictyon*, a general similarity is to be seen. However, *Hydrodictyon* has an even higher K:Na ratio (15 or 20:1, as against 5:1), and a very considerably lower actual Na, Ca, and Mg, than has *N. clavata*. On the other hand, it has about the same amount of sulfate. This may represent a characteristic of fresh water plants; if so they are in marked contrast to marine plants, which exclude sulfate. Permeability and electrical mobility studies of the sulfate ion in the two cases might be of interest therefore.

SUMMARY

Analysis of the cell sap of *Hydrodictyon patenaeforme* Pocock, from California indicates the usual marked accumulation of potassium, which is 4000 times as concentrated as in the surrounding pond water. Small amounts of sodium and calcium were found. Chloride makes up about three-fourths of the anions, with a very high sulfate, and much lower bicarbonate concentration accounting for most of the remainder. Electrical conductivity and osmotic studies indicate that the analyzed elements are ionized, and account for most of the sap's osmotic pressure. pH is 5.5 to 6.0.

The analytical procedure was designed to determine as many of the cations as possible on one small sample.

Hydrodictyon is a large multinucleate cell belonging to an order (Chlorococcales) new to permeability and accumulation studies.

THE CHEMICAL NATURE OF GROWTH FACTORS REQUIRED BY MOSQUITO LARVAE

II. Pantothenic Acid and Vitamin B_6

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When the larvae of the yellow fever mosquito, *Aedes aegypti*, are grown under sterile conditions (1), they may be shown to require accessory growth factors which they normally obtain from living microorganisms. Two of these factors are thiamin and riboflavin (2). If the larvae are supplied with protein, thiamin, and other dietary constituents in the form of a suitable amount of heat-killed yeast, they do not grow unless the yeast is further supplemented with adequate amounts of both riboflavin and a partially purified fraction of liver extract designated as the calcium filtrate (2). The experiments to be reported in this paper have been concerned with attempts to discover the nature of the essential substances present in the calcium filtrate fraction.

Methods

The larval growth tests were conducted in the manner previously described (2). All tubes received 0.3 ml. of a washed, killed yeast suspension in distilled water and either riboflavin or the flavin-purine complex (2). As formerly, all operations were carried out under strictly sterile conditions and appropriate tests for sterility were made. The growth index $N \times \frac{1}{T}$ (N = percentage of larvae reaching the 4th or last larval instar within 10 days, T = the average time in days required by these larvae to reach the 4th instar) has again been used. Under optimum conditions, at 28°C., $N \times \frac{1}{T} = 100 \times \frac{1}{4} = 25$.

Preparation of the Growth Factors

The preparation of the flavin-purine complex has been detailed in a previous paper (2).

The Barium Precipitate and the Barium Filtrate.—The charcoal elute already described (3) was concentrated to a small volume (1 ml. = 100 gm. of liver) and was

* Y. S. acknowledges financial aid from the Markle Foundation.

precipitated with 10 volumes of 95 per cent alcohol and 10 volumes of ether. The mixture was left in the cold room overnight. It was filtered. The filtrate was concentrated to remove alcohol and ether and diluted with distilled water so that 10 ml. = 100 gm. of liver. Then it was extracted 4 times with an equal volume of butyl alcohol.

The butyl alcoholic extracts were combined and concentrated *in vacuo* to dryness. The residue was taken up in 95 per cent alcohol (5 ml. per 100 gm. of liver). The alcohol extract was filtered. The filtrate was neutralized with alcoholic solution of $Ba(OH)_2$. The precipitate was filtered. The precipitate was termed B.P.S. and the filtrate was termed B.F.

Before use, B.P.S. was suspended in water, treated with sulfuric acid to precipitate the barium, and filtered. The pH of the filtrate was adjusted to 5.8–6.0.

Fractions Rich in Pantothenic Acid.—Fraction 355 was the same fraction the preparation of which was described under the number S-13 in a paper dealing with the growth requirements of rats (4).

Fraction 362-6 was a zinc salt. Starting with fraction 355, the ether extract was neutralized with ZnO and concentrated to dryness. The alcohol insoluble portion of the precipitate was discarded. The alcohol soluble portion was concentrated to a small volume and was precipitated with 50–60 volumes of acetone. The precipitate was filtered and dried. The precipitate was found to contain about 20 per cent zinc, 20–30 per cent nicotinic acid amide, 10–15 per cent uracil. This material, before use, was decomposed with H_2S, the sulfide filtered off, and the pH of the filtrate adjusted to 5.8–6.0.

Starting with 362-6, a barium salt of pantothenic acid was prepared. The zinc from 10 gm. of fraction 362-6 was removed by H_2S. Part of the nicotinic acid amide and uracil were removed by adsorption on English fullers' earth. Part of the inert organic acid in the filtrate was removed by the use of the thallium salt. The filtrate was decomposed with HCl, neutralized with $Ba(OH)_2$, and precipitated with acetone. The pantothenic acid was regenerated by the addition of enough $\frac{N}{1}$ sulfuric acid to precipitate the barium. The barium sulfate was filtered off and the pH of the filtrate adjusted to 5.8–6.0.

The pantothenic acid content of the three preparations was determined by their effect on the NY 5 streptococcus, which requires 1 γ of pantothenic acid for maximum growth (5).

RESULTS

Effects of the Barium Precipitate and Barium Filtrate Fractions of Liver Extract

In the presence of killed yeast and flavin-purine complex or riboflavin, the barium precipitate alone supported very little growth of the mosquito larvae. The barium filtrate fraction, at concentrations down to such that 100 ml. of solution contained the material derived from 40 gm. of liver, gave good growth and the addition of B.P.S. had but little effect. At lower concentrations of B.F. the growth fell off markedly and a clear effect of

the further addition of B.P.S. could be demonstrated (Table I). Since the barium precipitate had to be supplied at a concentration such that 100 ml. contained the amount from 150 gm. of liver, it is quite possible that this fraction was merely furnishing more of the same essential substances furnished by the barium filtrate.

Effects similar to that of B.P.S. could be obtained with yeast nucleic acid, at concentrations down to about 20 mg. per 100 ml. of medium, and

TABLE I

Effects of the Barium Filtrate and Barium Precipitate Fractions of Liver Extract, and of Yeast Nucleic Acid and Glutathione in the Presence of the Former Fraction

Concentration						$N \times \frac{1}{T}$
As gm. of liver from which was derived amount of fraction present in 100 ml. of culture fluid			Mg. per 100 ml. culture fluid			
Flavin complex	Barium filtrate	Barium precipitate	Yeast nucleic acid	Glutathione	Riboflavin	
50*	20	0	0	0	0	8.3
50	20	150	0	0	0	19.3
50	0	150	0	0	0	5.4
50	20	0	20	0	0	16.2
50	20	0	10	0	0	13.8
50	25	0	0	0	0	11.2
50	25	150	0	0	0	15.0
50	25	0	33	0	0	17.6
50	25	0	27	0	0	16.7
0	30	0	0	0	0.08	2.1
0	30	0	0	13	0.08	17.6
0	30	0	0	6.7	0.08	11.5
0	30	0	0	3.3	0.08	8.1
0	30	0	10	0	0.08	13.8
0	30	0	10	13	0.08	13.8

* Corresponds to 10 mg. per 100 ml. of culture medium.

with glutathione at a concentration of 13 mg. per 100 ml. (Table I). However, the addition of yeast nucleic acid and glutathione together gave no better growth than either substance alone.

Several partially purified fractions from liver extract could also replace the barium precipitate fraction. However, since the barium filtrate fraction alone seemed to contain the major part of the essential growth factors, attention was concentrated on trying to replace it by combinations of the various pure substances isolated from it. A great many experiments were performed, in all of which B.P.S. was supplied at a concentration such that 100 ml. of medium contained the material derived from 150 gm. of liver.

The results were extremely variable and in no case was satisfactory growth obtained. A general impression was gained that β-alanine, adenosine, and nicotinic amide[1], but especially β-alanine, had a slight favorable effect. While tubes containing as supplement only the barium precipitate gave $N \times \frac{1}{T}$ values of 5 or less, those containing B.P.S. together with β-alanine, adenosine, and nicotinic amide frequently gave $N \times \frac{1}{T}$ values over 5 and in one case as high as 12. It became obvious, nevertheless, that further experimentation with the known substances isolated from the barium filtrate would be futile.

Pantothenic Acid and Vitamin B_6

Since the liver preparations containing the mosquito growth factors also contained vitamin B_6 (6), some tests of this substance were performed. It was found that in the presence of killed yeast, flavin-purine complex, and barium precipitate fraction, both crystalline "Factor 1"[1] (7) and crystalline vitamin B_6[1] (8, 9) had a slight but definite growth-promoting effect. Nothing even approaching optimum growth was obtained.

Progress in the purification of pantothenic acid (10, 11), its recognition as one of the factors essential for the growth of chicks (12, 13) and rats (14, 15), and its presence in liver preparations (4) active as mosquito growth factors, led to trials of its effect on the growth of *Aedes aegypti* larvae. Three preparations, two (355 and 362-6) containing about 20 per cent pantothenic acid and one, a barium salt, 60 per cent pantothenic acid and 20 per cent barium, all markedly promoted growth of the larvae. When added to killed yeast and flavin-purine complex, the regenerated zinc salt alone gave fairly high values of $N \times \frac{1}{T}$ and these could be brought almost to the maximum (22.2) by the further addition of vitamin B_6. The optimum concentration of zinc salt in either case was about 0.02 per cent, representing a pantothenic acid concentration of about 4 mg. per 100 ml. The other crude pantothenic acid preparation (355) supported very little growth unless supplemented with vitamin B_6. In the presence of the latter, $N \times \frac{1}{T}$ values of 15 to 20 were obtained when that amount of pantothenic acid was present in 100 ml. which was derived from 100 gm. of liver.

[1] We are grateful to Dr. H. Adkins of the University of Wisconsin for sending us some nicotinic amide, to Dr. S. Lepkovsky for a sample of his crystalline Factor 1, and to Merck and Co. for supplies of vitamin B_6-hydrochloride.

The most significant results were obtained with pantothenic acid regenerated from its barium salt (Table II). In the presence of killed yeast, flavin-purine complex, vitamin B_6, and glutathione, growth was very slight and no larvae reached the 4th instar. The further addition of the regenerated barium salt of pantothenic acid enabled growth to proceed to the adult stage. As is evident from Table II, a concentration of 6.7 mg. of barium salt per 100 ml., corresponding to about 4 mg. of pantothenic acid per 100 ml., gave the best growth. The value of $N \times \frac{1}{T}$ was 15.8 in one experiment and 21.4 in another, and almost all of the larvae reached the

TABLE II

The Effects of Pantothenic Acid and Vitamin B_6

Concentration mg. per 100 ml. culture fluid						$N \times \frac{1}{T}$	Adults from 6 larvae		Average days to reach adult stage
Flavin complex	Riboflavin	B_6	Regenerated barium pantothenate*	Glutathione	Nicotinic amide		♀	♂	
10	0	0	6.7 (4)	0	0	1.9	0	0	
10	0	1.3	6.7 (4)	0	0	13.8	1	2	12
10	0	0	6.7 (4)	13	0	0	0	0	
10	0	1.3	6.7 (4)	13	0	15.8	4	1	14.5
10	0	1.3	6.7 (4)	13	8	17.6	2	4	14.5
0	0.04	0	6.7 (4)	0	0	0	0	0	
0	0.04	1.3	6.7 (4)	0	0	11.2	1	0	22
10	0	1.3	0	13	0	0	0	0	
10	0	1.3	13.3 (8)	13	0	9.2	1	2	17.5
10	0	1.3	6.7 (4)	13	0	21.4	2	4	13.5
10	0	1.3	3.3 (2)	13	0	9.6	1	2	18

* Number in parentheses gives the approximate concentration of pantothenic acid.

adult stage in 13 to 14 days. If glutathione was omitted, growth was always not quite as rapid as in its presence and fewer of the larvae reached the adult stage. If B_6 was omitted growth was very slow and few or none of the larvae reached the 4th instar (Table II). If riboflavin was substituted for the flavin-purine complex, growth proceeded at a much slower rate (Table II). A few of the larvae nevertheless reached the adult stage in this medium in which the liver extract was replaced entirely by known substances.

The addition to a medium of killed yeast, flavin-purine complex, vitamin B_6, pantothenic acid, and glutathione, of various other substances such as yeast nucleic acid, β-alanine, adenosine, tryptophane-betaine, nicotinic amide, inosine, etc., had no effect except in the case of nicotinic amide which in some experiments slightly accelerated growth (Table II).

Vitamin B_6, which has been recently synthesized and shown to be 2-methyl-3-hydroxy-4,5-di-(hydroxymethyl) pyridine (16), was absolutely essential for growth (Table II). Whether a more or less nearly pure preparation of pantothenic acid was used, the optimal concentration of vitamin B_6 was 1.3 mg. per 100 ml. of medium.

DISCUSSION

The major accessory factors, essential for the growth of mosquito larvae and supplied by liver extract in a medium of killed yeast in liver extract, appear to be riboflavin, pantothenic acid, and vitamin B_6, with glutathione and nicotinic amide having a lesser growth-stimulating rôle. It may be that the killed yeast, which supplies enough thiamin, also supplies almost enough glutathione and nicotinic amide. The experimental results show clearly that other essential substances supplied by liver extract remain to be discovered. These are present in the barium filtrate and more especially in the flavin-purine complex. While killed yeast, vitamin B_6, pantothenic acid, and glutathione, with flavin-purine complex, gave excellent growth, the same materials with pure riboflavin gave considerably slower growth. The rôle of yeast nucleic acid is difficult to evaluate. Although it could be substituted for the barium precipitate fraction in the presence of the barium filtrate, it had no effect on the growth obtained in the presence of flavin complex or riboflavin plus vitamin B_6, pantothenic acid, and glutathione.

At present, one can safely conclude that *Aedes aegypti* larvae require, as accessory growth factors, thiamin, riboflavin, pantothenic acid, and vitamin B_6; probably glutathione and nicotinic amide, and certainly other as yet unknown substances present in yeast and in liver extract. Since pantothenic acid consists of β-alanine in amide linkage with a hydroxy acid (11), one may account for the observed slight favorable effects of β-alanine by assuming that some individual larvae have very limited powers of synthesizing pantothenic acid if they are supplied with β-alanine, as is the case with certain strains of bacteria (23, 24).

Older work on the growth factor requirements of insects has been previously noted (2). The flies *Lucilia sericata* (17) and *Drosophila melanogaster* (18) and the beetle *Dermestes volpinus* (19) require cholesterol. Both *L. sericata* (20) and *D. melanogaster* (21) require thiamin, and the latter also requires riboflavin (21). Tatum (22) has recently found that *Drosophila* larvae required, in the presence of all the known vitamins, three additional factors present in yeast autolysate. Two of these could be separated from each other by precipitation with barium hydroxide in alcoholic solution,

a separation which suggests that they may be the same as the barium precipitate and filtrate fractions required by *Aedes aegypti* and that one of them may be pantothenic acid. Tatum (22) also showed that nicotinic acid is essential for the growth of *Drosophila* larvae.

As more work is done, it becomes increasingly obvious that insects require the same growth factors of the vitamin B complex group as do vertebrates. This is not surprising, since numerous species and strains of bacteria and yeast also require the same substances for growth (5, 23–26). Other species and strains of bacteria and other microorganisms can synthesize one or more of these factors (23, 24, 27). Microorganisms of this latter group under natural conditions supply mosquito larvae with their essential growth factors.

SUMMARY

The larvae of *Aedes aegypti* grew normally under sterile conditions in a medium consisting of killed yeast, flavin complex, or riboflavin, and two fractions derived from liver extract and designated as the barium filtrate and barium precipitate. The latter fraction could be replaced by yeast nucleic acid or by glutathione.

The larvae also grew at an almost optimal rate in a medium consisting of killed yeast, flavin-purine complex, vitamin B_6, pantothenic acid, and glutathione. All of these constituents except the glutathione were absolutely essential. Replacement of the flavin-purine complex by pure riboflavin resulted in slower growth, but nevertheless some larvae reached the adult stage.

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EFFECTS OF HEXYLRESORCINOL ON NITELLA

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The effects of hexylresorcinol[1] show some resemblance to those of guaiacol,[2, 3] as might be expected in view of their chemical similarity.

But interesting differences exist. Guaiacol increases the mobility of Na^+ (u_{Na}) and leaves that of K^+ (u_K) unchanged, but hexylresorcinol decreases[4] both mobilities. Hexylresorcinol 0.0003 M brings about as much negative change in P.D. as does 0.03 M guaiacol. The latter is not toxic in brief exposures (up to 5 minutes) but with the same exposure hexylresorcinol shows some toxicity at 0.003 M since some cells recover their normal P.D. when replaced in 0.001 M NaCl but others fail to do so. At 0.0003 M complete recovery may occur in a few minutes but such cells do not always live well afterwards.

Fig. 1 shows the effect of applying[5] 0.0003 M hexylresorcinol.[6] The curve

[1] $C_6H_3(OH)_2(C_6H_{13})$.

[2] $C_6H_4(OH)(OCH_3)$ 1:2.

[3] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417; 1939–40, **23**, 171.

[4] It is assumed that the mobility of Cl^- (v_{Cl}) remains unchanged since v_{Cl} is taken as unity. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715.

[5] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) until used. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 315) unless otherwise stated.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). The temperature varied from 20 to 29°C.

Unless otherwise stated there was no indication of injury.

Two spots, *D* and *E*, were recorded but the record of *E* is omitted in the reproductions given here. Both *D* and *E* were connected through the galvanometer to a spot *G* at the end of the cell. Any change at *G* would be revealed by simultaneous changes in the records of *D* and *E*: no such change occurred in the records here given.

After an exposure of some minutes with 0.0003 M hexylresorcinol at *D* and 0.01 M NaCl at *E* there was sometimes a sudden loss of P.D. at *E*, as though hexylresorcinol had passed from *D* to *E*, a distance of 1 cm. where the cell was surrounded by moist air: since hexylresorcinol is surface-active this may have played a rôle.

[6] The hexylresorcinol was kindly donated by the firm of Sharp and Dohme of Glenolden, Pa.

records the difference between two spots, *D* (in contact with 0.01 M NaCl) and *G* (in contact with 0.01 M KCl which reduces the P.D. approximately to zero). At the start, *D* had a positive[7] P.D. of about 65 mv. When the reagent was applied at *D*, the curve, after a latent period of about 32 seconds, rose slowly, indicating a loss of P.D. The latent period and the slow rise of the curve recall the effects of guaiacol on *Nitella*,[8] *Halicystis*,[9] and *Valonia*.[10]

The average duration of the latent period was about 25 seconds with 0.0003 M hexylresorcinol: it became shorter as the concentration was increased.[11] It seems possible that this is due, in part at least, to the time necessary for the reagent to penetrate through the protoplasm to the inner protoplasmic surface, *Y*, which is the chief seat[12] of the P.D.

When the depression of the P.D. reaches a certain point it may call forth an action current,[13] as seen in Fig. 2. This has also been observed when the P.D. is depressed[14] by KCl and has been explained as due to the discharge from a neighboring region.

A few cells gave curves like that shown in Fig. 3. Here the record shows the difference in P.D. between two spots, *D* and *G*, both in contact with 0.01 M NaCl. On applying hexylresorcinol at *D* the curve fell and then rose, indicating an increase in the positive[15] P.D. followed by a decrease. The promptness of the initial change indicates that the effect is on the outer surface of the protoplasm. The subsequent rise of the curve appears to be of the usual sort, probably involving penetration to the inner protoplasmic surface.

[7] The P.D. is called positive when positive current tends to flow from the vacuole across the protoplasm to the external solution.

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417.

[9] Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 707.

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13. In this case the P.D. becomes more positive.

[11] The values varied from 3 to 180 seconds.

[12] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215. The upward movement of the curve also occurs when we apply 0.0003 M hexylresorcinol dissolved in distilled water to a spot previously in contact with distilled water. Hence it does not depend on the salts in contact with the external surface.

[13] This may be propagated along the cell but usually is not. It is more apt to be propagated when the latent period is short.

[14] Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541.

[15] A change in the positive direction is produced by guaiacol in *Valonia*. Osterhout, W. J. V., *J. Gen. Physiol.*, 1936–37, **20**, 13.

In order to get at the cause of the positive change determinations were made of the concentration effects of NaCl and KCl. With normal cells

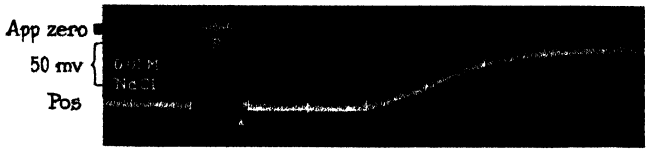

FIG. 1. Shows the effect of hexylresorcinol 0.0003 M. At the start the recorded spot (*D*) in contact with 0.01 M NaCl had an outwardly directed (positive) P.D. of about 65 mv. When its contact with the solution was broken the curve jumped to *F*, the free grid of the amplifier. Contact was then made (marked by arrow) with 0.01 M NaCl + 0.0003 M hexylresorcinol and after a latent period of about 32 seconds the curve slowly rose approximately to zero. (The zero is labelled "App. zero" since it is only an approximation.)

Two spots, *D* and *E* (1 cm. apart), were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M KCl (which reduced the P.D. at *G* approximately to zero). Any change at *G* would be shown by simultaneous changes in the records of *D* and *E*. No such change occurred. The record of *E* (not shown here) shows that no changes occurred at *E*.

Vertical marks 15 seconds apart.

The cell was freed from neighboring cells and kept 5 days in Solution A at 15 ±1°C. The experiment was performed at 21°C.

FIG. 2. Shows an action current induced by hexylresorcinol. At the start the spot recorded (*D*) in contact with 0.015 M NaCl had a positive P.D. of about 95 mv. When its connection with the solution was broken the curve jumped to *F*, the free grid of the amplifier. It was then placed in contact with 0.015 M NaCl + 0.0003 M hexylresorcinol. After a latent period of about 27 seconds the curve rose gradually until an action current occurred.

Two spots, *D* and *E*, were recorded (the record of *E* is not shown), both were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M KCl (which reduces the P.D. approximately to zero). The records show that there was no change of P.D. at this spot or at *E* during the experiment.

Vertical marks 15 seconds apart.

The cell was freed from neighboring cells and kept for 3 days at 15 ±1°C. in Solution A. The experiment was performed at 23°C.

the following values were obtained. The average concentration effect[16] of NaCl (0.01 M followed by 0.001 M or *vice versa*) varied from 20 to 41 mv.

[16] The dilute solution is positive in the external circuit.

(depending on which lot of cells was measured). The corresponding values for KCl[17] are 28 to 49 mv.

During exposure to hexylresorcinol 0.0003 M the concentration effect[18] of NaCl falls off and may approach zero.[19] This indicates that the mobility of Na^+ (u_{Na}) is approaching that of Cl^- (v_{Cl}).

When this happens, NaCl has less tendency to lower the P.D. and the result is a downward (positive) movement of the curve, as seen in Fig. 3. But if the change in u_{Na} is delayed this effect may be masked by the tendency of the curve to rise as the result of other changes. This happens in

FIG. 3 Shows an effect of 0.003 M hexylresorcinol. At the start the spot recorded, *D*, in contact with 0.01 M NaCl, had an outwardly directed (positive) P.D. of about 103 mv. When its contact with the solution was broken the curve jumped to *F*, the free grid of the amplifier. It was then placed in contact with 0.01 M NaCl + 0.003 M hexylresorcinol. The curve fell, indicating an increase in P.D. and then rose approximately to zero which is here taken as though 0.01 M KCl were at *G*.

Two spots, *D* and *E*, were connected through the galvanometer to a spot *G* at the end of the cell in contact with 0.01 M NaCl. The record of *E* (not shown here) shows that no change occurred at *E* or *G* during the experiment.

Vertical marks 5 seconds apart.

The cell was freed from neighboring cells and kept for 5 days in Solution A at 15 ± 1°C. The experiment was performed at 29°C.

many cases, as seen in Figs. 1 and 2. The duration of the positive dip therefore varies considerably.

The concentration effect of KCl also falls off during exposure to hexylresorcinol indicating that u_K is approaching v_{Cl}.

Before exposure the average potassium effect, *i.e.* 0.01 M KCl followed by 0.01 M NaCl, amounts to from 53 to 76 mv. (depending on which lot

[17] When 0.001 M KCl is followed by 0.01 M, action currents may occur which make the change in P.D. unduly large. *Cf.* Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541. In all cases the change was made from 0.01 to 0.001 M.

[18] *I.e.*, the effect of substituting 0.001 M for 0.01 M, both solutions containing 0.0003 M hexylresorcinol.

[19] Although the test may be made after the curve has risen approximately to zero the spot is not dead for it recovers its normal P.D. when the hexylresorcinol is removed unless the exposure has been prolonged beyond 5 minutes and even then there is recovery in many cases.

of cells is measured). During exposure to the reagent this also falls off. This indicates that K^+ and Na^+ are becoming more alike in respect to mobility[20] or partition coefficient or both.

It may be added that the variability of the cells used in these experiments was unusually great. This was due in part to the fact that they were collected at intervals throughout the year and covered an unusual range of seasonal variations.

A recent paper by Höber and coworkers[21] states that hexylresorcinol reversibly depresses the resting P.D. of frog muscle and of frog nerve. They ascribe this to a dispersing effect on the colloids of the surface. It is probable that hexylresorcinol produces structural changes in *Nitella* but the nature of these alterations requires further investigation.

SUMMARY

In some ways the effects of hexylresorcinol on *Nitella* resemble those of guaiacol but in others they differ.

Both substances depress the P.D. reversibly and both decrease the potassium effect.

Hexylresorcinol decreases the apparent mobility of Na^+ and of K^+. Guaiacol increases that of Na^+ but not of K^+.

The action of hexylresorcinol is more striking than that of guaiacol since 0.0003 M of the former is as effective as 0.03 M of the latter in depressing the P.D.

It is evident that organic substances can change the behavior of inorganic ions in a variety of ways.

[20] Regarding this see footnote 3.

[21] Höber, R., Andersh, M., Höber, J., and Nebel, B., *J. Cell. and Comp. Physiol.*, 1939, **13**, 195.

THE COMPOSITION OF FLUIDS AND SERA OF SOME MARINE ANIMALS AND OF THE SEA WATER IN WHICH THEY LIVE

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Knowledge of the chemical composition of body fluids and blood sera of the common marine invertebrates of North America is surprisingly scarce, although for European species the data are more complete (Bialaszewicz, 1933; Bethe and Berger, 1931; Robertson, 1939). When, recently, we had the opportunity of analyzing numerous species we found a parallel series of analyses had been completed by Homer W. Smith but never published. Professor Smith has kindly permitted us to include his data here and thus to make the present report considerably more complete than it otherwise could have been.[1]

Material and Methods

For all the species studied, care was taken to avoid contamination of the fluids by sea water or by other fluids of the animal, and to use only fresh intact animals in good condition. After drying the outside surface of each animal the fluids were allowed to drip into clean containers or were removed by pipette or hypodermic syringe. Where clotting occurred, the fluids were vigorously whipped to remove the clot. In a few cases

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* The fluids studied by W. H. C. were collected at Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine, during the summers of 1938 and 1939 and analyzed at Rutgers University. Preliminary reports were made by Cole and Kazalski (1939) and by Parker and Cole (1940). For assistance in preparing the samples we are indebted to Miss Barbara Parker and for analyzing them to Dr. J. D. Neuss. For financial assistance during 1939–40 we are indebted to the Permanent Science Fund of the American Academy of Arts and Sciences.

[1] The fluids studied by H. W. S. from echinoderms, worms, *Homarus*, and *Myxine* were collected and analyzed at the Mt. Desert Island Biological Laboratory during the summer of 1927 and those of *Homarus*, *Callinectes*, and *Limulus* in New York during the winter of 1927–28. Professor H. W. Smith expresses his indebtedness to the staff of the New York Aquarium for supplying live and acclimatized material; to the Elizabeth Thompson Science Fund during 1927–28 for financial assistance; and to Herbert Silvette for aid in the analyses.

the clots were analyzed separately in addition to the serum. If not analyzed immediately, about 1 ml. of toluene was added to each 100 ml. of fluid as a preservative.

Those analyses marked HWS (Table I) were made by the methods described by Smith (1929), while in those marked WHC the methods were as follows: chloride by the method of Wilson and Ball (1928) with the addition of nitrobenzene as suggested by Caldwell and Moyer (1935); sulfate by the microgravimetric determination of $BaSO_4$ using the filter stick technique; calcium by the method of Clark and Collip (1925), except that the precipitate of calcium oxalate was separated by filtration instead of centrifugation; magnesium by the method of Greenberg and Mackey (1932) using the calcium-free filtrate; sodium by the colorimetric method of Salit (1932) except that the precipitate was separated by filtration instead of centrifugation; potassium by the colorimetric method of Shohl and Bennett (1928) using filtration to separate the precipitate. Studies on the copper, nitrogen, and hemocyanin content of *Homarus*, *Cancer*, and *Limulus* will be reported later.

Where both CO_2 and pH determinations were made (HWS) the body fluid was removed without exposure to air into a syringe sealed with paraffin oil. In the case of small animals the analyses were made of pooled samples of blood from several specimens. In larger species (*Asterias*, *Solaster*, *Echiurus*, *Homarus*, and *Limulus*) individual animals were used. Those animals analyzed by HWS from "sea water" and "harbor water" respectively in New York had been in the New York Aquarium for several months and were more or less acclimatized. Although the sea water of the aquarium is constant in composition, the harbor water is continually pumped from off shore and varies in composition with the tide. The pH of the fluids studied by WHC was measured by the glass electrode and that of fluids studied by HWS by the quinhydrone electrode.

The freezing points were determined by the Beckman thermometer on pooled samples which had been in the refrigerator not more than 24 hours (WHC), or by a Heidenhain thermometer immediately after collection (HWS).

DISCUSSION

Discussion of the data will be facilitated by considering first the animals living in "full strength" sea water, and secondly, those living in diluted sea water or harbor water. Normal or full strength sea water may be arbitrarily defined as water containing 450 millimoles or more of chloride ions per liter.

Animals from Sea Water of "Normal" Concentration (≡ Chlorinity of 450 or More; Tables I and II)

The freezing points of the fluids of worms, echinoderms, and of *Venus* agreed well with that of sea water, indicating an isotonic condition. This conclusion agrees with other data in the literature, for a discussion of which the reader is referred to Krogh (1939).

Botazzi (1897), Macallum (1909–10), Schlieper (1935), and Pearse (1932) have reported that in the crab, *Limulus*, and the lobster the body fluid is hypertonic to sea water, while isotonicity has been reported by

Fredericq (1903–04), Garrey (1905), and Dailey, Fremont-Smith, and Carroll (1931). Since in our experience six different pooled samples each of *Homarus* and *Limulus* collected in three summers were consistently hypertonic we conclude that this is the normal condition. Our data are less extensive for the crabs, *Callinectes* and *Cancer*, but these also indicate hypertonicity.

The pH of all body fluids was distinctly below that of sea water. There was evidence that the pH of the sera of lobster, crabs, *Limulus*, and *Myxine* varied less among different individuals than did the pH of the blood and body fluids of the echinoderms, worms, and *Venus*. Furthermore, the amount of CO_2 present in fluids of the worms, Arthropoda, and *Myxine* was considerably greater than in the echinoderm fluids (excepting *Strongylocentrotus*), the CO_2 content of which was almost identical with that of sea water.

In accordance with procedure preferred by oceanographers, based primarily on the ease and accuracy of the chloride determination, the electrolyte composition is presented in terms of chlorinity; *i.e.*, millimoles of ions per liter divided by the millimoles of chloride ions per liter of fluid (Table I). For sea water at Salsbury Cove and New York the values obtained by HWS and WHC agreed closely with those reported by Clarke (1911) and Thompson (1936) for pure sea water. The small differences between the values of Thompson and those in Table I for potassium (HWS) (0.0179 *vs.* 0.0188 and 0.0204), and for sulfate (HWS) (0.0516 *vs.* 0.0629 and 0.0609) are perhaps due to the normal variation in the concentration of those ions in near-shore sea water, which has not yet been thoroughly mixed with open ocean water. Such variation could be caused by varying amounts of decomposition of small animals and plants, the former giving rise to excess sulfate and the latter to excess potassium.[2] The consistently low values for magnesium (WHC) for Maine sea water and for the fluids of animals from Maine (0.0977 *vs.* 0.0687, 0.0663, etc.) were probably due to some unknown systematic error in the analytical procedure for magnesium. The data of HWS are in closer agreement with the composition of Atlantic sea water. For the diluted sea waters from Delaware Bay and New York Harbor, the concentrations of potassium, calcium, and sulfate relative to that of chlorine were significantly different from those of pure sea water, a result to be expected in harbor waters (Clarke, 1911).

Although the actual ionic concentrations of the fluids give important information, the data are more easily interpreted by considering the ionic

[2] The marked accumulation of potassium by marine plants may be illustrated by *Valonia* (Osterhout, 1926–27).

concentrations of the fluids in relation to those in the external medium. The ratio of concentration inside to that outside furnishes evidence concerning the equilibria established with respect to the several ions. Such ratios are given in Table II, where the subscripts *i* and *o* refer to concentrations in the fluid and in the surrounding sea water respectively. Similar comparisons have previously been made for some of the ions in several invertebrates (Duval, 1925; Bethe and Berger, 1931; Dailey, Fremont-Smith, and Carroll, 1931; Bogucki, 1932; Bateman, 1933). In view of the latitude of analytical error and of variation between individuals, ratios between 0.9 and 1.1 should probably be taken to indicate uniform distribution between the internal and external medium. Ratios less than 0.9 indicate exclusion, and ratios greater than 1.1 indicate accumulation of ions relative to the external medium.

We find that the animals examined here may be classified into four

TABLE I

*Freezing Points, pH, and Composition of Some Marine Animal Fluids and of the Sea Water in Which the Animals Lived**

Species and habitat	Fluid	pH		$-\Delta^\circ$		mM/liter Cl		Chlorinity ratios in mM/liter										mM/liter CO_2
								Na/Cl		K/Cl		Ca/Cl		Mg/Cl		SO_4/Cl		
		HWS	WHC	HWS	WHC	HWS	WHC	HWS	WHC	HWS	WHC	HWS	WHC	HWS	WHC	HWS	WHC	HWS
Sea water, Salsbury Cove—Maine		—	8.10	—	1.759	483	492	0.861	0.931	0.0188	0.0173	0.0195	0.0197	0.1040	0.0687	0.0629	0.0516	2.15
Sea water—New York		—	—	1.850	—	525	—	0.859	—	0.0204	—	0.0293	—	0.0985	—	0.0609	—	2.15
Cucumaria frondosa—Maine	Coelomic	7.30	7.80	—	1.750	487	501	0.862	0.910	0.0199	0.0147	0.0192	0.0178	0.103	0.0663	0.0612	0.0509	2.14
Cucumaria frondosa—Maine	Ambulacral	—	7.75	—	1.749	—	494	—	0.962	—	0.0158	—	0.0180	—	0.0628	—	0.0514	—
Asterias vulgaris—Maine	Coelomic	7.20	7.54	—	1.762	488	505	0.846	0.911	0.0196	0.0164	0.0192	0.0178	0.104	0.0608	0.0615	0.0503	2.13
Chirodota laevis—Maine	Coelomic	7.00	—	—	—	488	—	0.861	—	0.0198	—	0.0209	—	0.113	—	0.0619	—	2.24
Solaster endica—Maine	Coelomic	6.90	—	—	—	488	—	0.861	—	0.0198	—	0.0196	—	0.102	—	0.0615	—	2.40
Echinarachnius parma—Maine	Coelomic	6.90	—	—	—	488	—	0.857	—	0.0189	—	0.0192	—	0.101	—	0.0611	—	2.24
Strongylocentrotus drobachiensis—Maine	Coelomic	7.20	7.84	—	1.776	488	510	0.861	0.904	0.0199	0.0188	0.0196	0.0173	0.0996	0.0608	0.0594	0.0496	6.00
Amphitrite brunnea—Maine	Blood	6.80	—	—	—	477	—	0.851	—	0.0273	—	0.0200	—	0.115	—	0.0646	—	5.0
Glycera dibranchiata—Maine	Blood	7.40	—	—	—	483	—	—	—	0.0199	—	0.0207	—	0.127?	—	0.0569	—	5.0
Echiurus pallasii—Maine	Coelomic	7.60	—	—	—	480	—	0.917	—	0.0263	—	0.0191	—	0.0885	—	0.0640	—	8.00
Venus mercenaria—Maine	Mantle	—	7.90	—	1.760	—	514	—	0.856	—	0.0144	—	0.0185	—	0.0486	—	0.0496	—
Homarus americanus—Maine	Serum	7.61	7.45	—	1.811	498	472	0.934	0.962	0.0172	0.0197	0.0214	0.0362	0.0095	0.0191	—	0.0106	5.12
Homarus americanus—New York	Serum	7.55	—	1.880	—	495	—	0.937	—	0.0181	—	0.0383	—	0.0186	—	0.0214	—	5.50
Cancer borealis—Maine	Serum	—	7.81	—	1.825	—	479	—	0.960	—	0.0213	—	0.0240	—	0.0457	—	0.0392	—
Callinectes hastatus—New York	Serum	7.55	—	1.932	—	480	—	0.958	—	0.0281	—	0.0410	—	0.0198	—	0.0238	—	7.0
Limulus polyphemus†	Serum	7.24	7.47	1.880	1.783	463	478	0.909	0.956	0.0311	0.0207	0.0346	0.0195	0.0584	0.0605	0.0445	0.0442	7.0
Myxine glutinosa—Maine	Serum	7.63	—	—	—	448	—	0.897	—	0.0203	—	0.0118	—	0.0502	—	0.0134	—	3.7
Delaware Bay water		—	8.01	—	1.336	—	319	—	0.878	—	0.0232	—	0.0169	—	0.0984	—	0.0514	—
Venus mercenaria—Delaware Bay	Mantle	—	7.65	—	1.369	—	373	—	0.799	—	0.0182	—	0.0298	—	0.0799	—	0.0515	—
Venus mercenaria—Delaware Bay	Blood	—	7.68	—	1.386	—	374	—	0.826	—	0.0184	—	0.0302	—	0.0778	—	0.0508	—
Limulus polyphemus—Delaware Bay	Serum	—	6.98	—	1.378	—	308	—	0.984	—	0.0282	—	0.0205	—	0.0799	—	0.0183	—
New York Harbor water		—	—	0.651	—	177	—	0.904	—	0.0252	—	0.0225	—	0.0910	—	0.0640	—	—
Limulus polyphemus—New York Harbor	Serum	7.46	—	1.01	—	265	—	0.981	—	0.0249	—	0.0208	—	0.0194	—	0.0169	—	10.8
Callinectes hastatus—New York Harbor	Serum	7.49	—	1.61	—	411	—	0.985	—	0.0204	—	0.0397	—	0.0730	—	0.0876	—	5.6
Homarus americanus—New York Harbor	Serum	7.56	—	0.945	—	270	—	0.859	—	0.0296	—	0.0518	—	0.0200	—	0.0148	—	5.1

* The total non-protein nitrogen in the sera in no case exceeded 20 mg. per cent and the urea nitrogen did not exceed half this value. Urea was almost entirely absent from *Homarus*, and of course was entirely absent in *Limulus* (Denis, 1922) the blood of which contains a high concentration of urease.

† Analyses by HWS were made on animals from sea water near New York; analyses by WHC on animals collected at Harpswell, Maine and kept in Salsbury Cove sea water for 3 months.

groups according to the composition of the body fluid compared to that of the external medium. The first group consists of the echinoderms and the clam, *Venus*, the fluids of which showed ionic ratios of 1.0 ± 0.1,

TABLE II

Ratios of Ionic Concentrations in Animal Fluids to Those in the Environmental Sea Water, Expressed in Millimoles of Ion Per Liter

Species and habitat	Fluid	Chlorinity of sea water in mM Cl/liter	$\frac{Na_i}{Na_o}$	$\frac{K_i}{K_o}$	$\frac{Ca_i}{Ca_o}$	$\frac{Mg_i}{Mg_o}$	$\frac{Cl_i}{Cl_o}$	$\frac{SO_{4i}}{SO_{4o}}$
* *Cucumaria frondosa*—Maine	Coelomic	488	1.003	0.965	0.957	0.991	1.013	0.993
* *Asterias vulgaris*—Maine	Coelomic	488	0.998	1.012	0.962	0.959	1.018	0.993
Chirodota laevis—Maine	Coelomic	483	1.010	1.060	1.085	1.100	1.010	1.007
Solaster endica—Maine	Coelomic	483	1.010	1.060	1.016	0.992	1.010	1.000
Echinarachnius parma—Maine	Coelomic	483	0.995	1.011	0.995	0.982	1.010	0.969
* *Strongylocentrotus drobachiensis*—Maine	Coelomic	488	1.008	1.097	0.981	0.944	1.023	0.981
Amphitrite brunnea—Maine	Blood	483	0.976	1.429	1.016	1.100	0.988	1.027
Glycera dibranchiata—Maine	Blood	483	—	1.055	1.064	1.228	1.000	0.927
Echiurus pallasii—Maine	Coelomic	483	1.057	1.385	0.975	0.850	1.023	0.994
Venus mercenaria—Maine	Mantle	492	0.961	0.871	0.979	0.740	1.045	1.004
Venus mercenaria—Delaware Bay	Blood	319	1.104	0.933	2.093	0.927	1.172	1.157
**Homarus americanus*—Maine	Serum	488	1.054	1.018	1.448	0.180	0.995	0.197
Homarus americanus—New York	Serum	525	1.028	0.838	1.231	0.181	0.943	0.330
Homarus americanus—New York Harbor	Serum	177	1.450	1.678	3.505	0.332	1.525	0.350
Cancer borealis—Maine	Serum	492	1.004	1.200	1.186	0.648	0.974	0.740
Callinectes hastatus—New York	Serum	525	1.020	1.262	1.279	0.184	0.914	0.356
Callinectes hastatus—New York Harbor	Serum	177	2.531	1.879	4.095	0.186	2.322	0.318
Limulus polyphemus—New York	Serum	525	0.933	1.346	1.039	0.522	0.882	0.644
Limulus polyphemus—Maine	Serum	492	0.989	1.112	0.949	0.828	0.972	0.824
Limulus polyphemus—New York Harbor	Serum	177	1.625	1.477	1.382	0.319	1.497	0.395
Limulus polyphemus—Delaware Bay	Serum	319	1.082	1.176	1.170	0.783	0.966	0.343
Myxine glutinosa—Maine	Serum	483	0.968	1.000	0.564	0.450	0.928	0.197

* These ratios were calculated from averages of the values of HWS and WHC from Table I.

excepting the unexplained low ratio of 0.74 for magnesium in the mantle fluid of *Venus*.

The second group includes the worms, two of which accumulated potassium (*Amphitrite* 1.4 and *Echiurus* 1.38), one accumulated magnesium (*Glycera* 1.2), and one excluded magnesium (*Echiurus* 0.85). Ratios for the other ions were 1.0 ± 0.1.

The third group includes the Arthropoda, all of which gave ratios of 1.0 ± 0.1 for the sodium and chloride ions, but excluded sulfate and magnesium; the lobster, *Homarus*, being especially efficient in this respect. As to the other ions, the following differences among the four species appear to be significant: *Homarus* serum contained on the average the same amount of potassium as the sea water but accumulated calcium; *Limulus* serum, on the other hand, contained the same amount of calcium but accumulated potassium; the crabs, *Cancer* and *Callinectes* accumulated both potassium and calcium. Ratios calculated from data on the European lobster, *Homarus vulgaris*, and crab, *Cancer pagurus*, reported by Robertson (1939) are closely similar to the ones given here, except for the calcium ratio in *Cancer* which is 1.47 instead of 1.19; and the potassium ratio in *Homarus* which is 1.45 instead of 0.98. Since a footnote suggested that the potassium content was "possibly too high" for *Homarus* the calculated ratio may also be too large.

The fourth group consists of the primitive chordate (*Cyclostomata*), *Myxine*, the serum of which showed ratios of 1.0 ± 0.1 for sodium, potassium, and chloride ions, but excluded about one-half of the calcium and magnesium ions and about 80 per cent of the sulfate ion. Although the freezing point depression was not recorded, the chlorinity of the serum indicated approximate isotonicity with sea water. Except for this difference *Myxine* serum resembled typical vertebrate sera much more closely than those of invertebrates.

The ratios for groups three and four showed unmistakable differential equilibria for at least three of the four ions: potassium, calcium, magnesium, and sulfate. In *Homarus*, for example, the average ratios were as follows: potassium, 0.928; calcium, 1.340; magnesium, 0.180; and sulfate, 0.264. The over-all distribution is, of course, the resultant of absorption and excretion but in the net indicates a physiologically preferential distribution across either the absorbing or excreting membranes. That such preferential capacities are physiologically significant among the invertebrates is sometimes overlooked, since the capacity of the fishes and higher aquatic vertebrates to regulate so efficiently the osmotic pressure of the body fluids, a capacity poorly developed among the invertebrates, overshadows the ionic composition of the lower forms. The unequal distribution of ions implies the expenditure of energy against a concentration gradient across the membranes involved. (Further evidence on this point may be found in the papers of Duval (1925), Bethe and Berger (1931), Dakin and Edmonds (1931), Bogucki (1932), and Bateman (1933).)

Animals from Diluted Sea Water (Chlorinity Less Than 450; Tables I and II)

Venus, *Homarus*, *Callinectes*, and *Limulus* collected from brackish water showed significant differences from specimens living in sea water. All the fluids were hypertonic to the external medium, even the mantle fluid of *Venus*. The relative hypertonicity of the arthropods was much greater than in sea water. It should be noted that the compositions of the mantle

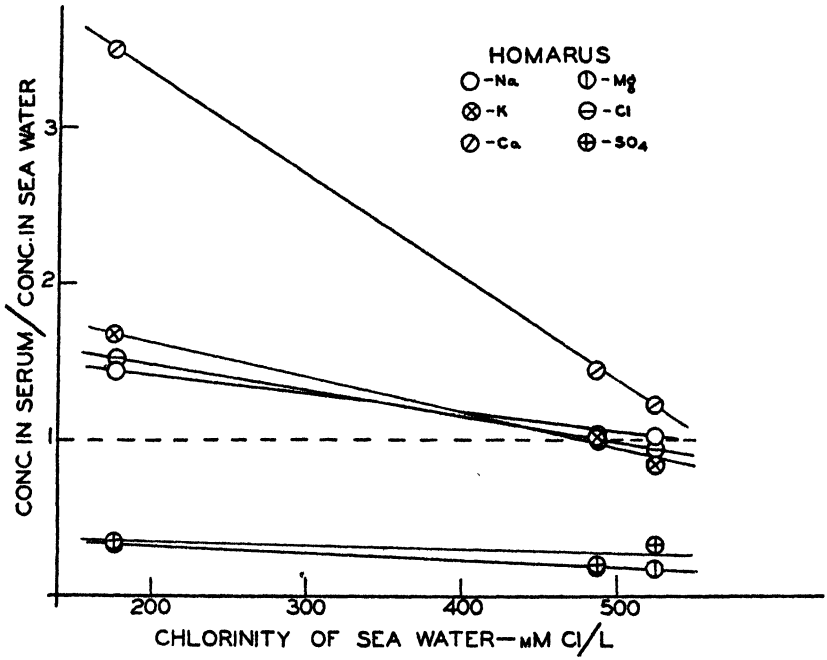

FIG. 1. Ratios of ionic concentrations in lobster (*Homarus*) serum to corresponding concentrations in environmental sea water plotted against concentration of chloride ions in the sea water.

fluid and of the blood of *Venus* were identical within the experimental error. Each contained more calcium than the Bay water. Since living conditions for the clam are less favorable in Delaware Bay than in pure sea water, it is likely that the increased calcium content is due to the solution of $CaCO_3$ from the valves, which was reported by Dugal (1939) in clams kept out of sea water for some time.

The increased hypertonicity of the Arthropoda was not caused by equally increased concentrations of the different ions. A graphical representation of the data for *Homarus* (Fig. 1) shows how the distribution of each ion changes independently when the animal moves from sea water into brackish

water. Ratios for magnesium and sulfate remain about the same in dilute sea water but the other ions accumulate in the following order: Ca>K>Cl>Na which differs from the order in sea water. Similar results are shown by *Callinectes* and *Limulus*. It has long been known (Krogh, 1939) that the fluids of marine invertebrates, isotonic to sea water become hypertonic to the external medium when the animals live in brackish water, but the differential accumulation of ions has not been reported. The possibility, however, was foreseen by Macallum (1926).

SUMMARY

1. The electrolyte composition, the pH, and freezing points of the fluids of several invertebrates and one primitive chordate are reported.

2. Fluids of the worms, echinoderms, and the clam *Venus* were isotonic with sea water; fluids of the Arthropoda were hypertonic to sea water.

3. The pH of all fluids was below that of sea water. In the Arthropoda and *Myxine* less individual variation in pH appeared than in the echinoderms and worms.

4. Ratios of ionic concentrations in the fluid to those in the sea water indicated (1) uniform distribution of ions between the internal and external media for the echinoderms and *Venus*; (2) differential distribution of potassium and magnesium in the worms; (3) differential distribution of sulfate, magnesium, potassium, and calcium in the Arthropoda; and (4) differential distribution of calcium, magnesium, and sulfate in *Myxine*.

5. The unequal distribution of ions implies the expenditure of energy against a concentration gradient across the absorbing or excreting membranes, a capacity frequently overlooked in the invertebrates.

6. The sera of the Arthropoda from diluted sea water showed higher concentrations of sodium, potassium, calcium, and chloride ions relative to the respective concentrations in the external medium than in normal sea water, and also showed different orders for those ions.

7. The increase in osmotic pressure of the sera of the animals moving into brackish water is caused by unequal accumulation of sodium, potassium, calcium, and chloride ions. Sulfate and magnesium ionic ratios do not change.

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EXPERIMENTS ON THE ADAPTATION OF ESCHERICHIA COLI TO SODIUM CHLORIDE

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INTRODUCTION

Since the isolation and pure culture study of microorganisms, investigations on the variability and adaptability of this biological material have frequently been undertaken with the hope of thereby contributing to some fundamental biological problems. A particular impetus to such studies was given by the development of the theory of evolution, the controversy between the monomorphistic and pleomorphistic viewpoints, and, more recently, by the discovery of adaptive enzyme formation. It seemed likely that an examination of the behavior of the outwardly undifferentiated microorganisms could contribute much towards a better understanding of the underlying principles of the phylogenetic evolution of higher forms.

While some definite results and interpretations have been obtained in studies on protozoa (Jollos (1), Jennings (2, 3), Hoare (4), Chatton and Tellier (5)), similar investigations with bacteria have yielded less definite, and sometimes controversial data. The reasons for this are not difficult to understand. Protozoa can be studied even as single individuals, which is virtually impossible in the case of bacteria. The experiments on the latter have, therefore, dealt with progenies of individuals rather than with the units themselves, so that the interpretations must be based on statistical analyses of growth curves, the morphological aspects of colonies, etc. Yet, the recent developments in the study of microbic variations (Beijerinck (6), Hadley (7), Mayer (8), Pinner and Voldrich (9), Doudoroff (10–12)) have clearly demonstrated that individuals in a pure culture may possess morphologically, physiologically, and biochemically different characteristics which can be transmitted to their offspring. On the other hand, the most important investigations on adaptation (Kluyver and Baars (13), Burke and coworkers (14, 15), Vaas (16), Karström (17), Stephenson and coworkers (18, 19), Knight (20), Lewis (21)) have not always made it possible to decide how far the observed variations were caused by the environment through a direct modification of the cells or through selection of individuals with certain pre-existing potentialities.

A serious difficulty in the application of the results of such studies to the interpretation of adaptability, mutability, and evolution of higher forms is the apparent absence of sexual phenomena in many of the microorganisms.

A detailed survey of the literature pertaining to these problems is found in the recent study of Vaas on adaptation of *Bacillus megatherium* to sodium chloride (16), to which the reader is referred.

The following report represents an exploratory study of the adaptation of *Escherichia coli* to an unfavorable environment. Sodium chloride was used in preference to other inhibitory agents because of the ease of its administration and the considerable literature on halo-tolerance and adaptation to saline media.

I

Preliminary Experiments and Standardization of Methods

1. Preliminary Experiments

The first experiments were designed to study the behavior of bacteria grown in ordinary fresh-water media on transfer to unfavorably saline nutrient solutions. The results can here be briefly summarized since Vaas (16) has recently published experimental data which are in complete agreement with my own. With an increase in the salinity of the medium the viable count of various fresh-water bacteria[1] remains constant until a certain NaCl concentration, varying with the species, has been reached; thereafter this count drops progressively with further increase. These results were interpreted to mean that a selective process was involved, some cells being better able to survive and reproduce under unfavorable conditions than others. The proportion of cells capable of reproducing in a medium with a certain salt concentration was fairly constant under a given set of conditions. This suggested a means of studying the "acclimatization" process, using as a criterion of adaptive changes the ability of the organisms to reproduce under unfavorable conditions, as measured by changes in viable counts made in defined saline media. This criterion of acclimatization is essentially the "success test" of animal and plant ecologists, described by Shelford.[2] A few investigators, *e.g.* Chatton and Tellier (5), and Vaas (16), have recognized the advantages of this criterion over those more commonly applied but basically different, such as (1) the death point or death time of organisms subjected to lethal concentrations or doses of the factors to which they had been acclimatized (Jacobs (23), Jollos (1),

[1] The bacteria used were: *Bacillus niger*, *Bacillus subtilis*, a species of *Pseudomonas* isolated from enrichment cultures containing inorganic salts and ethylene glycol, and two strains of *Escherichia coli*.

[2] Shelford, V. E., Laboratory and field ecology, Baltimore, The Williams & Wilkins Co., 1929, 99.

etc.), and (2) the metabolic rate of organisms in an unusual or unfavorable environment (Kluyver and Baars (13), Stephenson and coworkers (18, 19)).

Our method depends on the assumption that cells capable of reproducing in saline media produce viable offspring, and can therefore be counted by the colony or dilution method technique. The fairness of this assumption has been demonstrated by the results reported by Vaas (16) as well as my own.

It was necessary to standardize a technique for determining the number of bacteria viable in various concentrations of salt so as to obtain constant and reproducible results. Plate counts were found entirely unsatisfactory when high concentrations of salt were used in the agar. After some research the use of plate counts was abandoned, except for determinations of viable counts with little or no salt, and instead, the much more tedious, and under ordinary conditions less accurate method of broth dilutions was adopted. (*Cf.* (24), (25), and (26[3]).) Counts obtained in fresh-water broth agreed very closely with those arrived at by the plate method, and, as evidenced by the various tables, this method gave satisfactory results also when saline broth was used. Although the absolute viable counts in the same salt concentration varied from experiment to experiment, duplicate counts from the same culture were always in satisfactory agreement. The significant changes in viable counts induced experimentally were far greater than the variation among comparable counts in duplicate experiments. An additional advantage of the dilution method is the fact that the counts are made under conditions more closely approximating those of the cultures in which the organisms were grown.

For the development of a "standard technique" it was necessary to determine the effect of factors which influence considerably the viability of bacteria in saline broth.

The complexity of the problem soon made it necessary to limit either the scope of the investigation or the number of organisms to be used. Inasmuch as it was expected that a more comprehensive study of a single strain would yield more fruitful results, the experiments discussed in the following sections were carried out with a strain of *Escherichia coli.* This organism was chosen because so much work has been published dealing with its physiology. It should, however, be pointed out that a number of other organisms may have advantages for the study of such adaptive processes which would tend to weaken the justification for the continued use of this common intestinal

[3] Buchanan, R. E., and Fulmer, E. I., Physiology and biochemistry of bacteria, Baltimore, The Williams & Wilkins Co., 1928, 1, 10.

commensal, whose chief claim to popularity seems to be its abundant occurrence in feces, sewage, and drinking water. The strain used was a stock culture, isolated at the Hopkins Marine Station some 3 years previously, and kept since on yeast agar slants.

2. *Hydrogen Ion Concentration*

The pH of the saline broth in which the dilution counts were made is an important factor affecting the viability of the organisms. (See Table I.) For this reason the pH of the medium was carefully controlled and a considerable amount of buffer used.

The results summarized in Table I were obtained by making dilution counts of a suspension from a 24 hour yeast extract culture in media with

TABLE I

Dilution Counts of a Culture of E. coli in Media with Different NaCl Concentrations at Various pH Values

Viable counts with	0 per cent NaCl	5 per cent NaCl	6 per cent NaCl
pH			
6.0		4.5×10^6	2.5×10^1
6.5		2.0×10^6	9.5×10^2
7.0	1.8×10^9	7.5×10^7	4.5×10^5
7.5		9.5×10^7	2.5×10^5
8.0		6.5×10^7	4.5×10^5

two different NaCl concentrations, and adjusted to various hydrogen ion concentrations with the aid of a glass electrode.[4]

An acid reaction of the medium combined with high salinity is unfavorable to growth. Sherman and Holm (32) showed that NaCl increases the tolerance of *E. coli* to hydrogen ions. This increased tolerance of microorganisms to various unfavorable influences in the presence of relatively low salt concentrations may be rather general (Baars (28), van Niel (29)). Yet, the results laid down in Table I are not in conflict with these observa-

[4] The general composition of the nutrient broth was as follows:

Distilled water	
Yeast autolysate	2.5 per cent by volume
KH_2PO_4	0.135 per cent
$Na_2HPO_4 \cdot 12H_2O$	0.78 per cent
NaCl	5 per cent and 6 per cent respectively
NaOH or HCl to desired pH	

The yeast autolysate was prepared according to the method described by Elema ((27), p. 80).

tions; the salt concentration was so great that in itself it exhibited a toxic effect. The additional burden of an unfavorable hydrogen ion concentration thus merely increased the inhibition.

Between pH 7 and 8 there was no significant difference in viable counts. Hence neutral media were used for subsequent experiments, since the buffering capacity of the phosphates is greater there than at higher pH values, and since the cultures in yeast extract become alkaline as a result of metabolism.

3. *Concentration of Nutrients*

Table II shows that in media with 6 per cent and 7 per cent NaCl more bacteria could develop if the concentration of nutrients was reduced.

TABLE II

Number of Bacteria Developing in Saline Broth with Different Concentrations of Yeast Autolysate

Viable counts with.....	6 per cent NaCl	7 per cent NaCl
Concentration of yeast autolysate in per cent by volume		
0.5	2.5×10^8	4.5×10^5
1.0	7.5×10^8	2.5×10^4
2.5	4.5×10^5	9.5×10^2
5.0	4.5×10^5	2.5×10^1
10.0	2.5×10^6	7.5×10^0

These surprising results would lead one to believe that in the presence of high concentrations of NaCl the yeast autolysate exerts an additional inhibitory influence. The possibility seems excluded that such an effect would be due merely to an increase in the total osmotic pressure of the solution. From the observed boiling point of undiluted yeast autolysate the concentration of dissolved substances was computed to correspond osmotically to less than 2 per cent NaCl. The difference in osmotic effect between 0.5 and 1 per cent yeast autolysate would thus amount to an increase corresponding to only 0.01 per cent NaCl. The counts in 2.5 per cent yeast autolysate with 6 per cent NaCl and in 0.5 per cent yeast autolysate with 7 per cent NaCl show that the number of viable organisms in these two media is the same. Yet, the increase in osmotic effect due to the difference in yeast autolysate concentration corresponds to at most 0.04 per cent NaCl. Also, in media with 6 per cent NaCl an increase in nutrient concentration from 2.5 per cent to 5 per cent causes no further drop in the

viable count, while in the presence of 7 per cent NaCl the same difference in yeast autolysate concentration results in a 40-fold decrease. If one then considers that the number of viable organisms in 7 per cent NaCl-0.5 per cent yeast autolysate decreases to about 0.002 of the original with an increase of the yeast autolysate concentration to 2.5 per cent, whereas a further doubling of the amount of nutrients in 6 per cent NaCl has no effect, it becomes apparent that the influence of the concentration of yeast autolysate on the viability of the bacteria in NaCl media is quite complex.

No further attempts were made to elucidate this situation, and for later experiments a yeast autolysate concentration of 2.5 per cent was chosen arbitrarily. It may seem that a lower concentration would have been advantageous; yet at such concentrations the total growth of the bacteria decreases considerably, and even the gross results become inconsistent, varying with the batch of yeast autolysate used.

4. *Aeration*

Observations on the reproduction of bacteria taken from a fresh-water medium and suddenly immersed in saline broth showed that aeration also is an important factor in determining how many individuals will reproduce in the unfavorable environment. (See also (30, 31).)

Dilution counts of bacteria from a 24 hour yeast extract culture were made in 7 per cent NaCl broth under the following conditions:

1. In test tubes slightly stirred to insure an even suspension of organisms only.
2. In test tubes vigorously stirred after inoculation, and then left undisturbed.
3. In bottles, providing a large surface of medium exposed to the air.
4. In similar bottles, constantly rotated to insure thorough agitation of the medium.
5. In test tubes, slightly stirred, as in (1), the contents of which were placed in bottles after 24 hours of incubation and constantly rotated as in (4) till the completion of the experiment.
6. In bottles, constantly rotated as in (4) for 6 hours, after which time the contents were transferred to test tubes, and left unagitated as in (1).

The results are shown in Table III.

It is apparent that aeration during the first few hours after inoculation considerably increases the number of bacteria capable of reproducing in the saline medium.

For viable counts in further experiments, where at times several hundred cultures had to be incubated simultaneously, it was impractical to use constant stirring, and we were limited to the use of test tubes. The tubes were stirred vigorously for 10 seconds after inoculation and then left undisturbed. Although maximum values would not be obtained with this

method, the expectation that comparable results would be obtained by the use of a uniform technique was proved to be correct by further tests.

5. *Standard Method*

The standard method adopted for the further experiments may be summarized as follows:

1. All culture media were made with distilled water, and contained 0.5 per cent by weight KH_2PO_4–Na_2HPO_4 Sörensen buffer at pH 7 and 2.5 per cent yeast autolysate by volume (27). When salt was used in the media, sufficient NaOH was added to bring the pH to 7.0–7.1 as determined with the glass electrode.

2. Ordinary viable counts were made on yeast agar plates (10 per cent yeast autolysate in tap-water with 2 per cent agar, 0.1 per cent K_2HPO_4, and 0.05 per cent $MgSO_4$). In some cases, 3 per cent NaCl was added to the agar (where indicated). This salinity

TABLE III

Aeration and Viability of E. coli in Saline Broth

	Viable count in 7 per cent NaCl broth
1. In tubes, not vigorously stirred	4.5×10^1
2. In tubes, stirred after inoculation	6.5×10^2
3. In bottles, not stirred	1.5×10^3
4. In bottles, constantly agitated	2.5×10^6
5. In tubes during 24 hrs., then agitated in bottles	7.5×10^1
6. Agitated in bottles for 6 hrs., then transferred to tubes	2.5×10^5

did not reduce the viable count when organisms were taken from a fresh-water medium (see Table IV) and was used in those cases where the bacteria had been grown in more concentrated saline broth and a sudden change to fresh-water medium was not desired.

3. Total cell counts were made with a Petroff-Hausser counting chamber.

4. Viable counts in saline broth were made by the dilution method. Ordinarily, three series of decimal dilutions of the culture in saline yeast extract medium were prepared. The tubes were thoroughly shaken for 10 seconds after inoculation and sealed with "parafilm" to prevent concentration of the solution by evaporation. The most probable number of viable cells was determined from the tables prepared by McCrady (25).

5. The cultures from which the viable counts were made were grown in aeration flasks; the air bubbled through the cultures was passed through a column of NaCl solution at the same temperature and of the same salinity as that of the cultures.

6. The initial inoculum for an experiment was standardized by making two successive transfers in aerated fresh-water broth, each incubated for 24 hours.

7. All cultures were grown at 35°C.

By using this standardized technique, the results yielded by duplicate determinations in any one experiment agreed quite closely. The following

experiment demonstrates this. A 24 hour fresh-water broth culture was used for the estimation of the number of cells viable in a fresh-water and a saline medium. Six independent determinations were made, each consisting of three series of dilutions in 6 per cent NaCl broth. The results showed a total viable count (fresh-water medium) of 2.4×10^9 organisms per cubic centimeter and 2.5×10^5, 2.5×10^5, 4.0×10^5, 4.5×10^5, 4.5×10^5, 6.5×10^5 bacteria per cubic centimeter viable in saline broth.

In view of the fact that the dilution method *per se* is considerably less accurate than the plate count method, and that the viability of *E. coli* in 6 per cent NaCl media is dependent upon so many factors, the individual variations are not surprising, and the results may be deemed satisfactory.

The agreement between viable counts obtained by the dilution and plate methods in fresh-water media, and in those containing 3 per cent NaCl is

TABLE IV

Comparison between Plate Counts and Dilution Counts without and with 3 per cent NaCl

	Exp. 1	Exp. 2
Total cell count (determined microscopically)	2.7×10^9	2.1×10^9
Viable count, plate method, no NaCl.....	2.4×10^9	1.9×10^9
" " dilution " " "	2.5×10^9	2.0×10^9
" " plate " 3 per cent NaCl.....	2.2×10^9	1.9×10^9
" " dilution " " " "	2.0×10^9	2.5×10^9

revealed by Table IV, representing two experiments performed on different days.

It appears that the variation between duplicate experiments is insignificant at low concentrations of salt. It becomes, however, progressively wider with the use of more concentrated saline broth, as may be seen in Table V and in subsequent experiments.

II

Individual Variation in Adaptability to Saline Media

1. Differential Counts in Various Salt Concentrations

It has been stated that the proportion of individuals in a fresh-water culture capable of reproducing in a saline environment becomes progressively smaller with an increase in salinity. Table V is presented in illustration of this phenomenon.

This relationship between the percentage of viable organisms and the salinity of the medium was practically constant in a large number of experi-

ments, provided that the bacteria were grown in fresh-water broth under standard conditions. The experiments discussed in the following pages were designed to study the various factors by which this relationship can be changed.

2. *Influence of the Developmental Phase of the Culture*

Vaas (16) has shown that the ability of *B. megatherium* to grow in saline media, as determined by the fraction of total viable cells in fresh-water medium able to produce colonies in saline agar, is a function of the developmental stage of the culture. The adaptability was lowest in the early logarithmic period and greatest during the early stationary phase.[5]

TABLE V

Relation of Viable Count to NaCl Concentration

NaCl	Viable count	
	Exp. 1	Exp. 2
per cent		
0	2.5×10^9	2.0×10^9
3	2.0×10^9	2.5×10^9
4	9.5×10^8	4.5×10^8
5	2.5×10^7	7.5×10^6
6	4.5×10^5	2.5×10^4
7	9.5×10^2	2.5×10^1
8	Less than 1×10^0	

To determine the relation between the "physiological state" and the viable counts in saline broth, 100 cc. of 2.5 per cent yeast autolysate broth were inoculated with 0.001 cc. of a 24 hour culture of *E. coli*. Samples were taken out after various intervals of time. Total cell counts and total viable counts on yeast agar plates were made, as well as dilution counts in 5 per cent and 6 per cent NaCl broth. Fig. 1 shows the composite curves for three such experiments.

The total cell counts agreed fairly closely with the total viable counts in fresh-water media for the first 24 hours. Thereafter, the total cell count increased slowly, becoming about 15 per cent higher at 48 than at 24 hours; the viable count remained constant. The reaction of the culture was neutral during the first 8 hours of incubation, then became alkaline, the pH being 7.3 after 24, and 7.8 after 168 hours.

The results obtained with *E. coli* are in perfect agreement with the findings of Vaas for *B. megatherium*. As judged by the ratios of viable counts in saline broth to those in fresh-water media, the ability of *E. coli* to grow in a saline environment is greatest during the early stationary and least

[5] For the terminology used see Henrici (33).

during the logarithmic phase. Since the cultures were started with organisms in the early stationary phase, or at the peak of adaptability, the ratio decreases during the so called "lag" period. Adaptability decreases with senescence of the culture.

The curves bear a striking similarity to those obtained for the thermal death of *Paramecium* (Doudoroff (34)), and show the same relation of the physiological condition of the cultures to the adaptability of the organisms as to their resistance to lethal agents, as shown particularly by Sherman and Albus (35), and by Robertson (36). It is now generally recognized that organisms are most susceptible to toxic agents during the logarithmic phase of development. That such "physiologically young cells" (Sherman and Albus) can least stand transfer to unfavorable conditions suggests an intimate relation between their ability to reproduce under these conditions and their resistance to sublethal doses of toxic agents as established by the usual criteria of death.

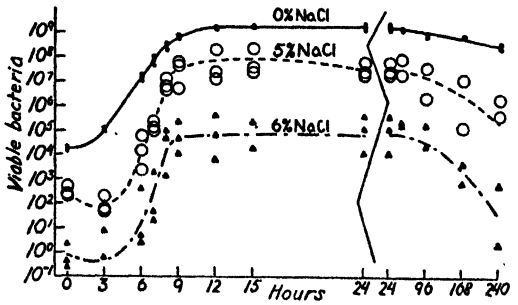

FIG. 1. Influence of the developmental phase of the culture on the adaptability of *E. coli* to sodium chloride.

3. *Selection or Acclimatization?*

It has been shown that a certain fraction of the total number of bacteria in a fresh-water culture could adapt themselves to an existence in a medium of given salinity. Growth in a concentrated saline environment involved a selection of those bacteria capable of reproducing under such conditions. That another factor besides the physiological state of the culture could be responsible for significant changes in the absolute value of this fraction soon became apparent.

If the salinity of the medium was raised gradually, more organisms from a 24 hour (stationary phase) culture were viable in saline broth than if the bacteria were suddenly immersed in the unfavorable medium. This agrees with numerous observations on the acclimatization of microorganisms to unfavorable environments by subjecting them to small doses of the toxic agent as reported in the literature (23, 5, 14, 15).

Equal portions of a 24 hour fresh-water culture were placed in each of four tubes and constantly aerated. At regular intervals concentrated NaCl solution (13.5 per cent NaCl, 0.5 per cent buffer) was added to each tube in such amounts as to increase the salinity of the mixture by 1 per cent NaCl steps with each addition. The intervals

between additions were 15, 30, 60, and 120 minutes in the four tubes respectively. In this manner, the salt concentration in the tubes was increased by steps of equal magnitude but of different duration. Viable counts were made in broth containing various concentrations of salt (from 3 per cent to 8 per cent). The samples for these dilution counts were taken after the organisms had been subjected to a NaCl concentration 1 per cent lower than that of the final test medium for a period of time corresponding to the duration of one step. Thus, the counts in 8 per cent NaCl broth were made 15, 30, 60, and 120 minutes after the seventh addition of salt to the tubes. Counts were likewise made using sudden immersion from the original fresh-water culture, corresponding to steps of no duration, and on salt-free agar plates.

The results are presented in Fig. 2. In Fig. 2A the number of bacteria viable in various saline media is plotted against the duration of each intermediate step, while in Fig. 2B the same data are entered with the viable count as ordinate and the concentration of salt as abscissa. It will be noted that all organisms were viable if placed directly into 3 per cent NaCl broth. Less and less cells could survive sudden transfer to more concentrated media. However, when the organisms were gradually subjected to the effect of higher salt concentrations greater numbers could reproduce in the unfavorable environment.

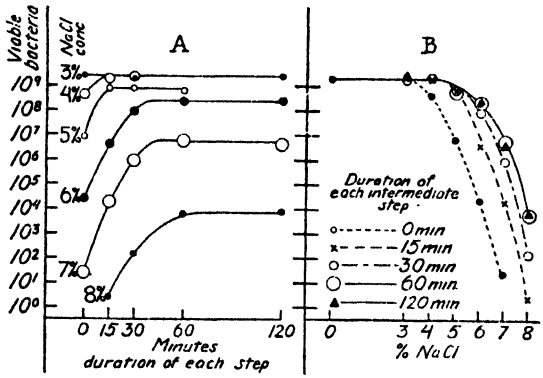

FIG. 2. Acclimatization of non-reproducing individuals of *E. coli* to sodium chloride by subjection to regularly increasing salt concentrations.

That this acclimatization, as expressed by changes in the developing fraction of bacteria, was a process of individual adaptation of the cells, in no way dependent on growth or selection, appears from the following considerations. The production of new cells would not be expected in a culture in the stationary phase when such a culture is subjected to unfavorable conditions without any addition of nutrients. No reproduction could be observed in the aeration tubes, either by plate counts, total cell counts, or determinations of the total volume of bacterial mass. If it is kept in mind that the initial discrepancy between the viable counts in fresh-water and in 4 per cent NaCl media disappeared as a result of the gradual increase in salinity, it becomes clear that, had this behavior been due to the formation of new cells in the mother culture, an increase in numbers would have

been readily detectable. Hence it seems that the gradual addition of salt has an effect on all rather than on some of the cells, and the increase in the fraction of the cells capable of reproducing in saline broth is merely an index of this effect, as measured by the hardiest or most "adaptable" individuals.

Fig. 2 shows further that the length of exposure to each concentration is of paramount importance. A maximum adaptation is obtained only by using exposures of sufficient duration. Shorter "steps" give lower values, while longer ones do not change the results of the viable counts. The minimum length of step giving maximum acclimatization depends on the final concentration in which the counts are made; the greater the salinity, the longer must be the duration of the intermediate exposures. Since the successive intermediate steps in each series were all of the same duration, it is impossible to determine whether the first or any of the later ones were responsible for the observed minimum period.

This type of acclimatization differs materially from the "race adaptation" brought about by the selection and perpetuation of those organisms capable of surviving, although in this case it has been detected and measured by the changes in the latter. It seems to be similar to the adaptation to salt in the protozoan, *Glaucoma piriformis* (5), and perhaps to the so called acclimatization to lethal temperatures in *Paramecium* (23).

III

Factors Influencing Non-Selective Acclimatization

1. Influence of Intermediate Salt Concentration

Fulmer (26)[6] as well as Chatton and Tellier (5) have shown that in order to induce acclimatization an exposure of the organisms to a single intermediate concentration of salts may be substituted for exposure to a number of small steps. It seemed desirable to find a single intermediate concentration of NaCl which would have an effect similar to that of a gradual increase in salinity.

Equal quantities of a 24 hour culture of *E. coli* grown in the absence of salt were mixed with equal volumes of saline solutions containing 0.5 per cent buffer at pH 7 and sufficient NaCl to bring the salinity of the cultures to 0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 per cent in the various tubes respectively. The mixtures were continuously aerated at 35°C. Samples were taken after various periods of time and counts made in 6.5 per cent NaCl broth. In those cases where the viable count in saline broth increased over that before the addition of salt, it was again found to reach a maximum after a short period of time.

[6] Buchanan, R. E., and Fulmer, E. I., Physiology and biochemistry of bacteria, Baltimore, The Williams & Wilkins Co., 1928, 2, 223.

The relation between the size of the intermediate step and the maximum number of organisms viable in 6.5 per cent NaCl broth at any time after the addition of salt is shown in Fig. 3. No reproduction of the bacteria could be detected.

As might be expected, no difference in adaptability of the organisms was observed among those receiving buffer only, those immediately brought up to the full concentration of the test medium (6.5 per cent NaCl), and those suddenly immersed in the saline broth without any intermediate treatment. Those bacteria, however, exposed to different salinities showed varying degrees of acclimatization, depending on the magnitude of the

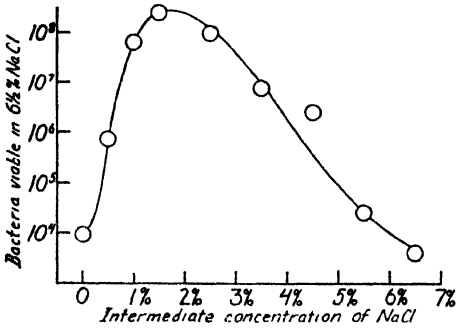

FIG. 3

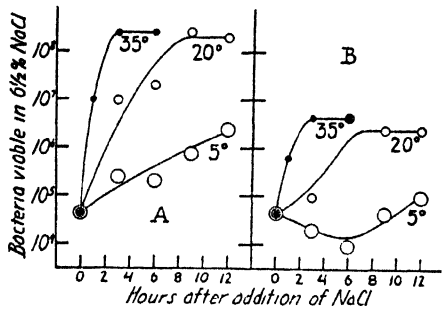

FIG. 4

FIG. 3. Acclimatization of *E. coli* to sodium chloride by subjection to a single intermediate salt concentration. Relation between the magnitude of the first step and the maximum number of bacteria becoming viable in 6.5 per cent NaCl broth.

FIG. 4. Effect of temperature on individual acclimatization.

A. 2.5 per cent intermediate NaCl concentration.

B. 4.5 per cent intermediate NaCl concentration.

intermediate concentration. It may be judged from this that the increase in viable counts was truly elicited by the salt and was not an artefact due to the dilution of the medium, aeration in saline medium, or some other such factor incidental to the addition of salt.

It appears that an intermediate step of about 1.5 per cent NaCl results in an optimal acclimatization. Very low concentrations are insufficient to cause maximal adaptation, while very large ones seem to be nearly as injurious to the bacteria as the saline broth in which the counts were made.

2. *Effect of Age of the Culture*

Old cultures were not as readily acclimatized to saline environment by the gradual addition of salt as were cultures in the early stationary phase of development.

Three samples were taken from a culture in the stationary phase 24 hours after inoculation, three after 48, and three after 96 hours. One sample from each period was used for immediate dilution counts in 6.5 per cent NaCl broth without previous treatment; the second and third samples received sufficient 13.5 per cent NaCl to bring the salt concentrations to 1.5 per cent and 4.5 per cent respectively. Portions of these were tested for viability in 6.5 per cent NaCl broth after exposure for varying lengths of time to these intermediate steps. The results are shown in Table VI.

It will be seen that as the culture ages and the number of bacteria capable of immediate reproduction in a saline medium decreases, the maximum number capable of being acclimatized also decreases. While in the case of a small intermediate concentration this decrease is comparable to that observed for the unacclimatized bacteria, in the case of a larger and less favorable intermediate step, the decrease is more pronounced. This suggests that increased susceptibility to injury by salt may be an earlier consequence of aging than the loss of "adaptive power" and may, in fact, be the cause of the latter.

TABLE VI

Effect of Aging on Acclimatization

Age of culture	Maximum viable count in 6.5 per cent NaCl broth		
	Intermediate concentration		
	None	1.5 per cent NaCl	4.5 per cent NaCl
hrs.			
24	9.5×10^8	2.0×10^8	4.5×10^6
48	4.5×10^8	7.5×10^6	4.5×10^3
96	9.5×10^6	7.5×10^4	2.0×10^1

3. *Effect of Temperature*

The rate of "individual acclimatization" depends on the temperature at which the organisms are subjected to increasing salt concentrations.

Portions of a 24 hour salt-free culture were introduced into aeration tubes, at 5°, 20°, and 35°C. respectively. Sufficient 13.5 per cent NaCl solution containing 0.5 per cent buffer was added to bring one set of cultures at the three temperatures to 2.5 per cent NaCl and the other set to 4.5 per cent NaCl.

After various intervals of time samples were taken from each tube and dilution counts made in 6.5 per cent NaCl broth. The results are shown graphically in Fig. 4, the data obtained with 2.5 per cent and 4.5 per cent intermediate concentrations being presented in parts A and B respectively.

Although the theoretical and practical limitations of the method used for determining the rate of adaptation made it impossible to plot the curves

accurately, it is apparent that acclimatization occurs more rapidly at higher temperatures than at lower ones.

Insufficiency of data and the difficulty of interpreting the results made the determination of a temperature coefficient impossible, although from the mere inspection of the curves a Q_{10} approximating 2 may be estimated. The difference in rates at different temperatures might be due to the combination of several factors responsible for "adaptive processes" on the one hand, and death of the bacteria on the other. This might explain the differences in the curves obtained with the more or less "optimal" intermediate salt concentration of 2.5 per cent and the less favorable one of 4.5 per cent.

TABLE VII

Reversibility of Acclimatization

	Time	Bacteria viable in 6.5 per cent NaCl broth	
	hrs.		
Before acclimatization		9.5×10^8	
After acclimatization	0	2.5×10^8	
		Bacteria resuspended in buffer	Bacteria resuspended in 1.5 per cent NaCl
"	1	2.5×10^5	7.5×10^7
"	3	4.5×10^4	2.5×10^8
"	6	2.0×10^3	9.5×10^7
"	20	4.5×10^1	2.5×10^6

A similar effect of temperature was demonstrated when adaptation was induced by gradually increasing the salt concentration.

4. *Reversibility of the Process*

Samples from a 24 hour culture in NaCl-free medium were subjected to 1.5 per cent NaCl for 4 hours, at which time the number of organisms capable of reproducing in 6.5 per cent NaCl had reached a maximum. A part of this culture was diluted with nine parts of distilled water containing 0.5 per cent buffer only. As a control, a similar portion was diluted with nine parts of buffered 1.5 per cent salt solution. Both suspensions were aerated at 35°C., and samples taken from each after various intervals of time for dilution counts in 6.5 per cent NaCl broth. No reproduction could be detected in the two suspensions either by plate counts or by determinations of the volume of bacterial mass. Table VII shows the results.

Within a few hours after the acclimatized organisms had been returned to fresh water, they had completely lost their increased ability to develop in saline media, as judged from the fact that the number of bacteria viable in 6.5 per cent saline broth had dropped below the original value obtained

before the conditioning process. At the same time those organisms remaining in 1.5 per cent NaCl solution showed little if any change in adaptive power. The relationship is even more striking after 20 hours, when in both cases a decrease in viable count in saline broth was observed. This decrease may be the result of a rapid senescence of the bacteria caused by the increased salinity combined with the dilution of the nutrients in the medium. It seems that such senescence would proceed equally rapidly in fresh-water and in 1.5 per cent NaCl solutions; the relative decrease in viable counts in the two media is practically the same between 6 and 20 hours. It thus appears that the processes responsible for the individual acclimatization are readily reversible.

That the decrease in the number of salt-viable bacteria is not due to death of the organisms is shown by the fact that the counts on salt-free agar not only failed to show an increase, but also revealed no significant decrease in the number of bacteria viable in fresh-water media. Thus, whereas the number of living individuals in the 1.5 per cent NaCl and in the NaCl-free solutions was the same, it was only in the former that they retained their ability to develop in the strongly saline medium. This, after all, is equivalent to stating that the process of individual acclimatization is reversible.

IV

Characteristics of Cultures Developing in Saline Media

1. Storage of Acclimatized Bacteria in Saline Broth

The experiments reported in the foregoing sections have dealt with the behavior of *E. coli* in salt-water media when the bacteria had been grown in NaCl-free environments, but without growth during the acclimatization period.

Before studying the effects of growth in a saline medium on adaptation it seemed desirable to determine what changes in adaptability might occur in organisms acclimatized to salt broth and left in this medium for a considerable period of time without opportunity for reproduction.

A 24 hour fresh-water culture was gradually brought up to 7 per cent salinity by seven 1 per cent steps of 1 hour duration, thus assuring maximum acclimatization of the bacteria to the saline environment. The resulting suspension was constantly aerated at 35°C. for 6 days and dilution counts in 7 per cent and 8 per cent NaCl broth, as well as plate counts on 3 per cent NaCl yeast agar were made after various intervals of time following the acclimatization. The results of two such experiments are plotted in Fig. 5A.

It is apparent that organisms acclimatized by the gradual increase in salinity remain in this state for a considerable period of time. No further significant increase in viable count in saline broth beyond the initially established maximum occurs. The curves also demonstrate that a part of the bacteria must have died in the salt medium, because the number of organisms viable in 3 per cent NaCl dropped somewhat, particularly during the early stages of the experiment. At the same time, the viable count in 7 per cent NaCl broth remained remarkably constant; no indication of a similar initial decrease was observed. The two curves thus show that the individuals which had become fully adapted are less readily killed.

Although the non-adapted cells die more rapidly than the adapted ones, yet the death rate is comparatively low. Hence, at all times the medium contains organisms incapable of reproducing in 7 per cent NaCl solutions but capable of development under more favorable circumstances, such as in a 3 per cent salt medium. These considerations show that there exists a real difference between the general physiological characteristics and responses of the individuals within a culture.

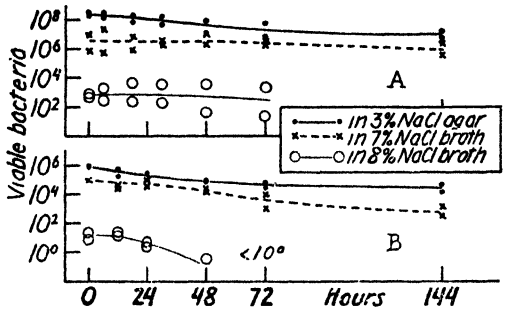

FIG. 5. Storage of acclimatized bacteria in saline broth.

A. Bacteria left at 35°C. in the medium in which they had developed, but with 7 per cent NaCl.

B. Bacteria placed in freshly prepared 7 per cent NaCl broth and kept at 0°C.

In the foregoing experiment the bacteria were left in the same medium in which they had developed, and to which the salt had been added. It was conceivable that fresh saline broth might have a different effect on the viability of the cells in NaCl-containing media, even if they could not divide in it.

To test this, bacteria were acclimatized as in the previous experiment, and left in the same medium for 18 hours at 35°C., when 99 parts of fresh broth containing 7 per cent NaCl and 0.5 per cent buffer were inoculated with one part of the culture, and the resulting suspension was kept constantly stirred in a water bath at 0°C. for 6 days. Samples were taken out at intervals and dilution counts made in both 7 per cent and 8 per cent NaCl broth. The results are shown in Fig. 5B.

While the low temperature prevents development in the freshly prepared saline medium, it has no appreciable effect on the ability of the cells to

multiply in saline broth at a more favorable temperature. The curves are quite comparable to those in Fig. 5A if it is remembered that the initial points in Fig. 5B correspond to those at 18 hours in the upper graph, and that the cultures have been diluted 100-fold.

The only significant difference between the curves in Fig. 5A and 5B is that the viable counts in 8 per cent NaCl broth in the last experiment seem to diminish more rapidly than in the previous one. This difference may be ascribed to the combined inhibitory effects of high salinity and low temperature on the adaptive process.

2. *Studies on Successive Transfers in Saline Broth*

It had thus been ascertained that a prolonged exposure of acclimatized bacteria to a saline environment in which no detectable development takes place does not result in a further increase in the salt-viable counts beyond the maximum reached during the initial acclimatization process. The development of *E. coli* in strongly saline media could now be investigated with a view to establishing whether or not the cells produced in the presence of salt would show different characteristics with respect to their adaptive powers from those previously studied.

Bacteria from a 24 hour fresh-water culture were exposed to increasing salt concentrations in seven 1 per cent steps of 1 hour duration each, and left in 7 per cent saline medium for 18 hours. Transfers from this adapted culture were made in yeast extract with 7 per cent NaCl, and successive subcultures in this medium started every 48 hours. During the development of the first, third, and fifth subcultures viable counts were made in 7 per cent and 8 per cent NaCl broth, as well as on 3 per cent NaCl agar plates. Counts on the second and fourth subcultures were made only 48 hours after inoculation. The composite results of two such experiments are plotted in Fig. 6A, B, and C, in which the curves represent the counts obtained in the first, third, and fifth subcultures respectively.

From Fig. 6A it will be seen that the number of bacteria viable in 7 per cent NaCl broth, although initially only a fraction of the total number of living cells, soon began to increase, this increase becoming logarithmic with time, although the total number of living organisms did not increase until the number of cells viable in 7 per cent NaCl medium had approached it. Thereafter the two curves coincided for a period of time extending slightly beyond the logarithmic phase.

Consequently only those cells originally viable in 7 per cent NaCl broth are capable of reproducing in this medium, while the others do not divide at all. The logarithmic order of growth indicates that all cells produced in saline medium can continue to reproduce in it. This is supported also by

the close agreement between the counts on 3 per cent and 7 per cent NaCl media in the subcultures. In addition, no discrepancy was observed between the viable and the direct microscopic counts during the logarithmic period in any of these experiments.

The 7 per cent NaCl-viable count falls below the viable count on 3 per cent NaCl plates with senescence of the culture. This suggests that even bacteria, grown in a strongly saline medium lose with age, their ability to propagate in such a medium although they are still capable of reproducing under more favorable circumstances.

An examination of the curves for the viable counts in 8 per cent NaCl broth in Fig. 6 A, B, and C reveals that they roughly parallel those for the counts in 7 per cent NaCl media. Consequently, the ratio of the number of organisms viable in 8 per cent to that in 7 per cent NaCl remains almost constant throughout. This warrants the conclusion that repeated transfers in saline broth do not cause any further acclimatization of the organisms to more saline environments.

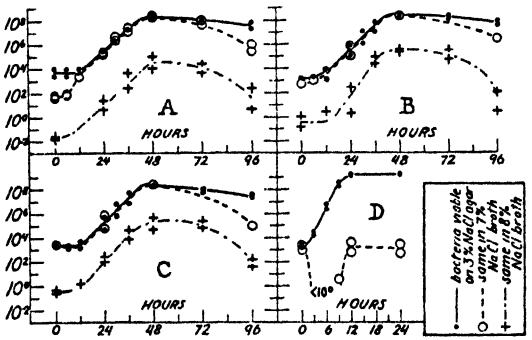

FIG. 6. Development and adaptability of *E. coli* in saline and salt-free broth.

A. 1st subculture in 7 per cent NaCl broth.

B. 3rd subculture in 7 per cent NaCl broth.

C. 5th subculture in 7 per cent NaCl broth.

D. Development in salt-free broth after three transfers in 7 per cent NaCl medium.

The ratio of 8 per cent NaCl-viable bacteria to the total number of bacteria present is higher in these experiments than it was after the "non-selective acclimatization" of cultures grown in NaCl-free media (*Cf.*, *e.g.*, Fig. 2). This might seem to imply a further acclimatization to higher salt concentrations, but a careful analysis of all the data obtained would rather support a different conclusion. The selective propagation of those cells capable of reproducing in 7 per cent NaCl media, combined with the ability of a constant fraction of such cells to develop in higher salt concentrations, would obviously yield the observed results. In view of the demonstration that the ratio between the numbers of bacteria viable in 7 per cent and in 8 per cent NaCl broth is practically constant, and that in 7 per cent NaCl broth no increase in 3 per cent NaCl-viable bacteria was found prior to an increase in 7 per cent NaCl-viable cells up to the initial number of the

former, the interpretation based on selection instead of individual acclimatization must be deemed more probably correct.

A number of investigators have obtained adaptation to unfavorable environments by culturing the organisms under conditions intermediate between the "normal" and the altered habitat. The results here reported indicate that the mechanism of such adaptation would consist of both individual acclimatization, which is independent of reproduction, and of selection by the environment of those cells endowed with a wider range of tolerance to normally unfavorable conditions.

Both the growth rate and the maximum crop are greatly diminished in the presence of high salt concentrations. This is clearly brought out in Table VIII, in which the minimum division times and the maximum crops

TABLE VIII

*Minimum Division Times and Maximum Crops in Fresh and Saline Media**

	Minimum division time	Maximum crop
	min.	
In salt-free medium	24	1.8×10^9
In 7 per cent NaCl broth:		
1st subculture	103	1.8×10^8
2nd "		1.6×10^8
3d "	104	1.7×10^8
4th "		1.9×10^8
5th "	103	1.9×10^8

* The figures represent means of two experiments in each case.

in saline and in salt-free cultures are compared. These findings agree with those of Eisenberg (37), Estor (38), Speakman, Gee, and Luck (39), and Vaas (16).

From the curves in Fig. 6A, B, and C, and particularly from Table VIII, it is clear that repeated transfers in the 7 per cent NaCl broth do not lead either to a faster growth of the bacteria or to a larger crop than can be obtained with the first saline culture. These observations further support the contention that such transfers do not yield bacteria with increasingly greater potentialities but simply a larger number of acclimatized cells by a process of selection.

The morphological characteristics of cultures in media without salt differed from those in concentrated saline broth. In non-aerated salt-water cultures the growth was stringy and mucoid; the organisms formed a sediment reminiscent of agglutinated bacteria. This was never observed in fresh-water cultures, where the medium became homogeneously turbid;

and even in old cultures, where precipitation had occurred, the deposit could readily be shaken up again to a uniform suspension. Similar differences, though less marked, could be observed with aerated cultures. Microscopic examination showed that in the NaCl-containing media the individual cells have a strong tendency to hang together; the bacteria are non-motile and form chains of appreciable length. During the logarithmic growth the organisms were distinctly longer in saline than in salt-free broth, but in the stationary phase the size of the bacteria was again reduced approximately to normalcy.

In very old NaCl-broth cultures the liquid became brown; such discoloration was never observed in fresh-water media.

3. Studies on the Behavior of Saline Broth Cultures upon Return to Fresh-Water Media

It has been shown that the manifestations of the individual acclimatization, induced by exposure of non-dividing cells to intermediate salt concentrations, rapidly disappeared when the cells were re-suspended in a salt-free environment.

Inasmuch as the experiments presented in the previous section have failed to demonstrate a detectable difference between cells thus acclimatized and new cells produced by frequent subcultures in concentrated NaCl media, it seemed probable that a reversibility of the adaptation process could also be proved for the latter.

After three transfers in 7 per cent NaCl broth, carried out as in the last experiment, a subculture was made in fresh salt-free medium. Viable counts on yeast agar as well as in 7 per cent NaCl broth were made during its development. The results of two such experiments are shown in Fig. 6D, while the mean minimum division rate and maximum crop under these conditions are presented in Table IX, together with those previously found in fresh-water and saline cultures.

From Fig. 6D it may be seen that there was hardly any lag phase and no death of bacteria following the transfer of saline-broth cultures to fresh-water medium. Yet, such phenomena might have been expected from the studies of Kluyver and Baars (13) and of Hoare (4). It must, however, be remembered that the potentialities of the organisms used by these investigators were apparently much more limited than those of *E. coli* and *B. megatherium* because after their cultures had been adapted to a new environment by a series of successive subcultures under progressively modified conditions the majority of the cells had become incapable of reproducing in the original one. Such limitations of potentialities might,

through continued selection, bring about a segregation of populations which are, in some physiological aspects, sufficiently different to suggest separate species.

It is also apparent from Fig. 6D and from Table IX that the division rate as well as the total crop immediately revert to their normal values for fresh-water broth. This definitely bars the possibility that the salt-tolerant cells of *E. coli* selected by the cultivation in a saline medium differ in their intrinsic reproductive powers from those incapable of dividing in the presence of high NaCl concentrations.

The viable count in 7 per cent NaCl broth, roughly equal to the total viable count at the beginning of the experiment, is seen to decrease so rapidly that 2 hours after inoculation it can no longer be computed. At the end of the logarithmic phase the number of bacteria viable in 7 per

TABLE IX

*Comparison between Growth Rates and Crops in Salt-Free and Saline Media**

	Minimum division time	Maximum crop
	min.	
In salt-free medium	24	1.8×10^9
In 7 per cent NaCl broth	103–104	$1.6–1.9 \times 10^8$
In salt-free medium after 3 transfers in 7 per cent NaCl broth	25	1.9×10^9

* The figures represent means of two experiments in each case.

cent NaCl again rises to the approximate value of similar counts made with ordinary fresh-water cultures never exposed to salt. This agrees with the findings of Vaas (16) and proves that whatever may be the difference between individuals viable and non-viable in saline broth, it certainly is not an hereditary distinction, and that the adaptation here studied can not even be classed as a "*Dauermodifikation*" as defined by Jollos (1).

Thus, the ability to reproduce in an unfavorably saline medium, acquired through the formation of new cells in such a medium, is lost as completely as that acquired by the exposure of non-dividing cells to intermediate salt concentrations after the return of the acclimatized bacteria to a salt-free environment.

V

GENERAL DISCUSSION AND CONCLUSIONS

The present treatise was meant as an exploration of but one of the many phases of the problem of adaptation of microorganisms to their

environment. It is apparent that many factors are involved in the adaptation of *E. coli* to saline media, each of which must be investigated in detail before it can be hoped that an understanding of the reactions involved will be reached.

The standard technique as developed here makes it possible to use the changes in the ability of bacteria to reproduce under altered conditions as a criterion of their "adaptive power" with nearly the same accuracy as can be attained in ordinary determinations of "death points" or "death times."

The experiments presented strongly support, if not conclusively prove, Vaas' hypothesis of a "fluctuating variation" among individuals in a pure culture with respect to their ability to develop in a modified environment. That the limits of such variability are dependent on the *milieu* has been demonstrated more clearly than in his own experiments, for it has been shown that the range of tolerance can be extended without any reproduction or selection occurring in the cultures. This individual acclimatization has been demonstrated to be reversible and influenced by temperature. That it does not merely involve the recovery of the bacteria from osmotic effects produced by the salt, as proposed by Chatton and Tellier (5) in explanation of the behavior of protozoa, seems likely from the observation that subjection of the organisms to a very low intermediate NaCl concentration may widen the range of variation to its maximal limits.

The developmental phase of the culture influences the ability of the bacteria to grow in saline media in much the same manner as it does the resistance to toxic agents in general, suggesting a close relationship between adaptability and resistance.

Some investigators have emphasized the applicability of the law of mass action in conjunction with due consideration of the size of bacteria to studies of death rates of bacteria, concluding that all the individuals had the same resistance. (For a detailed discussion of these problems see Rahn (40, 41), Holwerda (42).) The experiments described here provide ample evidence of the actual occurrence of variation among the individuals with respect to their adaptive ability; hence the possibility that variation within a population would influence its statistical "death rate" may not be disregarded.

The selection by the medium of individuals possessing a wider range of potentialities has also been demonstrated in the present investigations. That the displacement of the "limits of variability" has no hereditary basis or consequence must be particularly emphasized.

By the analysis of viable counts and growth curves it has thus been

possible to separate the processes involved in the adaptation of *E. coli* to a new (saline) environment into two components, namely: acclimatization which apparently does not involve reproduction, and selection of individuals with the greatest potentialities.

The methods outlined in the present work for separating the two factors are perhaps not the only available nor the simplest means of attacking the problem, but the results seem to justify the expectation that they may be used with equal success for the study of a variety of related problems.

I wish to express my sincere gratitude to Dr. C. B. van Niel, whose valuable advice I have sought on numerous occasions, and whose unfailing enthusiasm has been a source of encouragement and stimulation.

SUMMARY

1. It has been shown that a fairly constant fraction of the total number of bacteria in a fresh-water culture of *E. coli* can reproduce on direct transfer to a saline medium with a definite NaCl concentration, as judged from the viable count determinations in such a medium.

2. The absolute value of this fraction depends on a number of factors other than the salt content of the test medium, such as the hydrogen ion and yeast autolysate concentrations, aeration, and the physiological condition of the bacteria.

3. A method for testing the degree and rate of adaptation of the bacteria to saline environment, depending on the analysis of changes in the value of the salt-viable fraction, was developed.

4. Maximum adaptability to saline environments was found during the early stationary phase of NaCl-free cultures. Low adaptability accompanied the logarithmic phase and the senescence of the cultures.

5. The limits of variation could be extended by treatment of non-dividing cells with gradually increasing concentrations of salt or by subjecting them to a single intermediate NaCl concentration. This acclimatization was independent of reproduction. The number of bacteria becoming capable of reproducing in a hitherto unfavorable environment increased with the period of exposure to intermediate salt concentrations until a maximum value was reached.

6. This maximum value was shown to depend on the salinity of the test medium, the age of the bacterial culture, and the method of preliminary treatment. "Optimal acclimatization" could be effected by subjecting the organisms to a single fairly low intermediate NaCl concentration.

7. The rate of the individual acclimatization process was shown to be greater at higher than at lower temperatures.

8. Acclimatized bacteria rapidly lost their increased ability to reproduce in saline media upon return to a salt-free environment, although no reproduction of the cells could be detected. This was interpreted as an indication that the processes involved are readily reversible.

9. Studies on the reproduction of *E. coli* in strongly saline broth indicated that only those cells originally acclimatized to the salt concentration of the medium could divide. All cells produced in such a medium could continue to reproduce. The propagation in the altered medium was not accompanied by any further acclimatization throughout five subcultures.

10. Both the division rate and the maximum crop of cultures in saline broth were considerably lower than of those in a fresh-water medium. No change in either occurred throughout five successive subcultures. The morphology of the organisms was also altered by the presence of salt.

11. The division rate, maximum crop, morphology, and adaptive power returned immediately to normal on re-transfer of bacteria grown in an NaCl-containing medium to "salt-free" broth.

12. The entire adaptive response of the bacteria to a considerable increase in the salinity of the environment could thus be separated into two components: an acclimatization, independent of reproduction, and a selection of those cells with the widest range of potentialities.

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THE RELATION BETWEEN FOVEAL VISUAL ACUITY AND ILLUMINATION UNDER REDUCED OXYGEN TENSION

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INTRODUCTION

Numerous studies have demonstrated that a diminished partial pressure of oxygen in the inspired air produces marked alterations in the functioning of the central nervous system (McFarland, 1932). All the tissues of the body, and particularly the nervous tissue, are extremely sensitive to anoxia and to variations in the concentration of carbon dioxide. Certain functions that involve the retina, morphologically a part of the brain and metabolically resembling it (Weinstein, 1932), also manifest extensive changes upon exposure to low oxygen tensions. It has not been possible, however, to determine to what extent the effects of anoxia on vision are due to alterations in the central nervous system, or in the sense organ itself.

Studies of dark adaptation have shown that alterations in light sensitivity offer an unusually delicate test of the initial effects of anoxia at partial pressures of oxygen where other sensory tests have failed to show reliable changes (McFarland and Evans, 1939). The dark adaptation curves (threshold against time) were progressively elevated on the log I axis with increasing oxygen deprivation, the magnitude of these changes being 0.10 of a log unit at 15.8 per cent O_2 (7,400 ft.), 0.22 at 13.7 per cent O_2 (11,000 ft.), and 0.40 at 11.7 per cent O_2 (15,000 ft.). Subjectively, there appears to be a general darkening of the visual field during anoxia. Subsequent exposure to oxygen results in a marked increase in the brightness of lights (Goldmann and Schubert, 1933). These changes are probably not concerned with the photochemical processes in the retina, but with the neural elements of both the retina and the central nervous system (Bunge, 1936–37; McFarland and Evans, 1939). In careful experiments on one subject, McDonald and Adler (1939) have recently reported that anoxia causes an equal elevation of rod and cone thresholds, whereas vitamin A deficiency produces a greater change in the rod threshold.

Changes in certain other visual processes have been observed under conditions of acute oxygen deprivation. Schubert (1932–33) and Gellhorn (1936) have observed a considerable decrease in visual intensity discrimination while inhaling 8 to 10 per cent oxygen. They used Masson discs, which provide a rather crude measure of this function. A decrease in critical fusion frequency for flickering light was reported at 10.6 per cent O_2 by Seitz (1940). Gellhorn and Spiesman (1935) noted a lengthening of the latent period of the negative after-images or even a complete absence of any after-images. Alterations in negative after-images were also observed by McFarland (1937) in subjects acclimatized to 17,500 ft. and 20,000 ft. in the Chilean Andes. The effects of anoxia on the central visual field have been studied by Evans and McFarland (1938). In experiments in a low oxygen chamber, they found that with progressive oxygen deprivation, beginning at a concentration corresponding to an altitude of 13,000 ft., there was a progressive widening of the angioscotoma (Evans, 1938) (projected defect related to the retinal perivascular spaces). The visual field was obliterated except for an area 8 to 10° about the macula. The measurements were made on a stereocampimeter with test objects of about 0.5 mm. diameter, at 190 mm. from the eye.

The effects of anoxia on peripheral visual fields have been studied by several investigators with somewhat contradictory results. Wilmer and Berens (1918) reported a narrowing of the fields for form and color, in all quadrants, at altitudes of 15,000 ft. and above, simulated in a low pressure chamber. Goldmann and Schubert (1933) found decrements in only the nasal and superior fields. Upon repetition of their experiments, however, Kyrieleis, Kyrieleis, and Siegert (1935) observed no changes in the fields beyond the limits of error. It is possible that these discrepancies were due to differences in experimental conditions and technique. Significant changes in color vision due to oxygen deprivation have been described by Vishnevskiy and Tsyrlin (1935), Velhagen (1936), and Schmidt (1937–38).

Previous investigations of visual acuity under low oxygen tensions have not been very conclusive. In none of them has the rôle of illumination been taken into consideration. Wilmer and Berens (1918), using a rebreather and a low pressure chamber, reported experiments on twenty-five normal males with the Ives visual acuity test object. They found no change in 60 per cent of their subjects, improvement in 12 per cent, and a decrease of an unstated amount in 28 per cent, from which they concluded that anoxia causes a decrease in visual acuity. On the other hand, Bagby (1921) also using a Henderson rebreather and the Ives test object, found

no significant change in visual acuity until just previous to collapse, when there was a marked deterioration not only of the sensory function but also of attention and ability to cooperate. More recently Berger and Bøje (1937) made a study of the ability of two emmetropic subjects to resolve[1] two squares while breathing air containing 8.7 per cent oxygen. Two methods were used: luminous squares on a dark field, and black squares on a white field. In spite of marked oxygen lack, they reported that the resolving power was unchanged or only slightly decreased while using the luminous squares, whereas with black squares on a white field a considerable decrease was found. The thresholds rose 30 per cent in one subject and 100 per cent in the other. The brightness of the background corresponded to a log I in photons of 2.875. They attributed the changes in this case largely to an alteration in the intensity discrimination threshold (*cf.* Gellhorn, 1936).

Visual acuity is dependent, among other things, upon the intensity of illumination. Uhtoff (1886, 1890) made the first thorough investigation of this problem over a great range of illumination using white and colored lights. A few years later Koenig (1897) made such comprehensive observations that his data have become classic. The most adequate and precise measurements of this relationship were described recently by Shlaer (1937–38). His apparatus was constructed so as to avoid certain variables which were uncontrolled in previous investigations, namely size of the pupil, distance of the test object, and an extensive surrounding field equal in brightness to that of the test field. The latter is necessary in all measurements involving a stationary state of adaptation of the eye.

The data of Shlaer, as also those of Koenig, when plotted as the logarithm of visual acuity (ordinate) against the logarithm of the intensity of retinal illumination (abscissa) (Hecht, 1937), are consistent with the theory that the retina is a double sense organ. Using white light, a discontinuity appears in the curve at a visual acuity = 0.16. All values below this are mediated by the rods, and those above by the cones. The two portions of the curve were shown by Shlaer to fit a theoretical equation derived by Hecht (1934) upon the simplest assumptions concerning a photoreceptor

[1] The word "resolve" is used in its technical sense meaning ability to discriminate *detail*. In the study of Berger and Bøje this detail was the space between the two squares. It cannot be assumed that "resolving power" as measured by these authors is synonymous with visual acuity. In an earlier publication they stated that their measurements with black squares were the more closely related to "visual acuity," as ordinarily determined. This method more closely approximates the conditions of our experiments than does that with luminous squares.

system. If red light, to which the rods are believed to be relatively insensitive,[2] is used instead, the rod portion is deleted and the data fall upon a continuous curve which represents a function of the cones alone, and also corresponds to Hecht's equation (Shlaer, Smith, and Chase). This is also apparent in Koenig's data when plotted as a double-logarithmic function (Hecht, 1937). Under most situations, the visual acuity mediated by the cones is probably of greater importance than that which involves the rods. For this reason, and also in order to simplify the treatment of our data, the experiments to be described were made with the use of red light rather than white.

Apparatus and Procedure

Visual Acuity Apparatus.—The apparatus we employed for the measurement of visual acuity at various intensities of illumination was that described by Shlaer (1937–38). The illumination was varied discontinuously in steps of approximately 0.3 of a log unit by means of neutral Wratten filters placed just within a 2 mm. artificial pupil. A No. 70 Wratten (red) filter was also placed at this point. The size of the test object could be varied continuously over a range of about 1:100, at a fixed distance of 1 meter from the eye. It was located in the center of a uniformly illuminated field 30° in extent. In our study it appeared as a black figure against a red background. The test object used was a Landolt broken circle, or C, in which the width of the line and the gap is $\frac{1}{5}$ the total outside diameter of the letter. It could be rotated about its optic axis so as to be presented in eight different meridians, 45° apart. The apparatus was placed in a room with black walls and ceiling, and the subject was shielded from extraneous light.

The test object was set by the experimenter so as to be too small for the subject to resolve it. The latter turned a knob, which caused the size of the object to increase, in steps of about 0.010–0.020 of a log unit of visual acuity. The subject paused between adjustments to observe the object, until he was able to report the meridian in which the opening of the C was located. If he was correct, the scale reading of the visual acuity was taken, and the test object was reset below the threshold for another determination. If the response was incorrect the meridian was changed and the subject allowed to proceed. The order in which the meridian settings were made was according to a predetermined pattern. Readings on the visual acuity scale were made to 0.001–0.002 of a log unit, depending on the part of the scale employed. With trained subjects successive determinations usually agreed within 0.030 of a log unit or better.

Low Oxygen Apparatus.—The various mixtures of oxygen and nitrogen were inhaled by the subjects from a rubber mask which covered the mouth and nose and fitted tightly against the face. Cylinders of these gas mixtures were prepared and their composition verified by analysis of samples in duplicate on the Haldane apparatus. The gases

[2] More precisely, as pointed out by Hecht (1937): "It is not that the rods do not function in red light; it is that in red light the cones have about the same intensity thresholds as the rods; but since the cones are closer together in the fovea than are the rods in the periphery, they can resolve smaller distances and therefore dominate the measurements by recording the correspondingly higher visual acuities."

entered a 50 liter Douglas bag from which they passed during inspiration through a one-way mercury valve to the mask. Upon expiration this valve closed and the expired gases passed through a flutter valve on the mask into the room. Changes from one gas mixture to another could be made by the experimenter without the subject being aware of it.

Subjects.—The subjects were eleven males, of sedentary occupation, all but two of whom were medical students. Their age range was 20-25 years with the exception of one (F. B.) who was 43. All were emmetropic and free of organic diseases. They were, on the average, in fair physical condition, the test having been made during the latter part of their summer vacation.

EXPERIMENTAL PROCEDURE

Two series of experiments were performed as follows:

Series 1 consisted in measurements of visual acuity over practically the entire range of illuminations mediated by the cones, while the subjects breathed normal air and while they breathed an oxygen mixture containing 10.34 per cent O_2. The partial pressure of oxygen in such a mixture corresponds to that at an elevation of 18,000 feet, according to Bureau of Standards data for constant temperature of 15°C. (*cf.* McFarland, 1938).

The two subjects whose data we present were first given an extensive amount of practice. They made observations at each of the light intensities used in the experiment over a period of several days, so that they exhibited no further learning and their measurements were quite consistent.

The actual experiment required about 4 hours. The subject was first dark-adapted for about 10 minutes, and measurements were begun at the lowest intensities. The subject kept his eye as close to the artificial pupil as possible. Before making observations at any given intensity he fixated the center of the illuminated field for 3 minutes so as to reach a stationary state of adaptation at this intensity. Four observations were made at each intensity, each usually requiring 1 to 3 minutes. If any one of these varied from the others by more than 0.030 of a log unit, it was rejected and another taken in its place; this step was rarely necessary. After 5 minutes of observation, the subject was allowed to rest for 1-2 minutes with his eyes closed. Such rest periods were followed by a 3 minute period of adaptation to the illuminated field before observations were resumed.

Having completed the observations with normal air at all the light intensities employed, the subject was allowed to rest for 20 minutes. Then administration of the low oxygen mixture was begun and the entire procedure described in the preceding paragraph was repeated in a similar manner.

Upon completion of the measurements under these conditions, 100 per

cent oxygen from a cylinder was delivered to the mask and the subject was again allowed to dark adapt for 10 minutes. This change in composition of the inspired gases was made without the subject's being aware of it. Visual acuity determinations were then made at the lowest light intensity which had been used during the tests with the low oxygen mixture.

Series 2.—Nine subjects were tested at two intensities of illumination. One was near the low end of the range for cone vision (log I in photons = $\bar{1}.159$) and the other, nearly 10,000 times as bright (log I = 3.120) or great enough to elicit nearly maximal visual acuity. Two low oxygen mixtures as described below, were employed.

The practice period of about 2 hours was first given in which the subjects were trained to make observations at these intensities. Practice was also given at an intensity about 0.4 of a log unit lower. (See below.)

The duration of each experiment was about 2½ hours. The same general procedure was observed as in series 1. After the measurements in normal air had been completed at the two illuminations, determinations were made under the following conditions and in the following sequence:

(*a*) 14.31 per cent oxygen, equivalent to 10,000 feet.
(*b*) 10.34 per cent oxygen, equivalent to 18,000 feet.
(*c*) 100 per cent oxygen, as a control.

Each gas mixture was breathed for 15 minutes before the measurements were begun. Since, as will be seen later, only slight changes occurred at the high illumination, this was employed only at 10.34 per cent oxygen. At least six readings were made at each point, depending on the rapidity with which they could be made, and on their consistency with each other.

RESULTS

Series 1.—The data[3] of two subjects are presented in Table I, and are plotted in Figs. 1–3. Each datum of log visual acuity represents an average of four measurements. In Figs. 1 and 2, where the description is by the photochemical stationary state equation (Hecht, 1934), with visual acuity taken as proportional to x^2, the theoretical curve has been superimposed on the normal air data, and then translated *horizontally* to the right along

[3] Definitions of units employed:

Visual acuity is expressed as the reciprocal of the angle, in minutes, subtended by the finest detail distinguishable, which here corresponds to the gap in the *C* used as the test object.

Retinal brightness is given in photons (Troland, 1916) and is expressed as external brightness in millilamberts times $10/\pi$ times pupil area in square millimeters (photons = millilamberts $\times$ $10/\pi$ $\times$ pupil area in square millimeters).

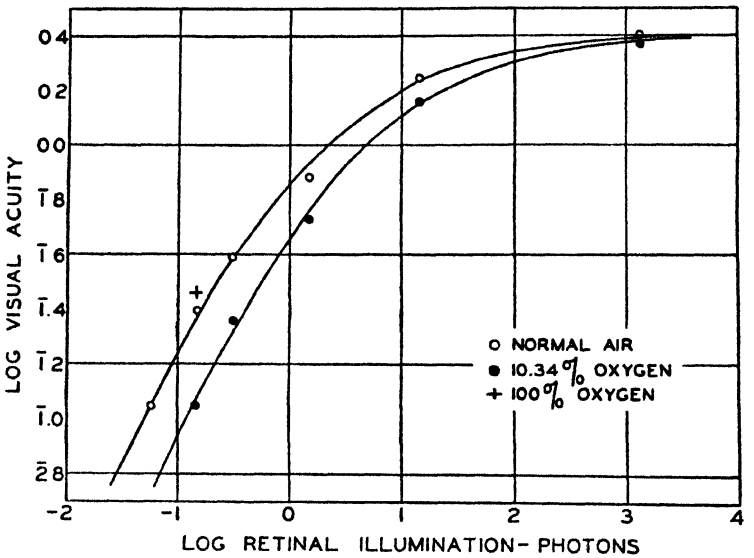

FIG. 1. The data for subject F. B. (Table I) showing the relation between log visual acuity and log retinal illumination with normal air and with 10.34 per cent oxygen. The curve corresponding to Hecht's stationary state equation (1) (see Discussion) has been superimposed on the normal air data; it has then been translated *horizontally* to the right to obtain a fit for the low oxygen data. The + represents a final control with 100 per cent oxygen, after completion of the measurements with 10.34 per cent oxygen.

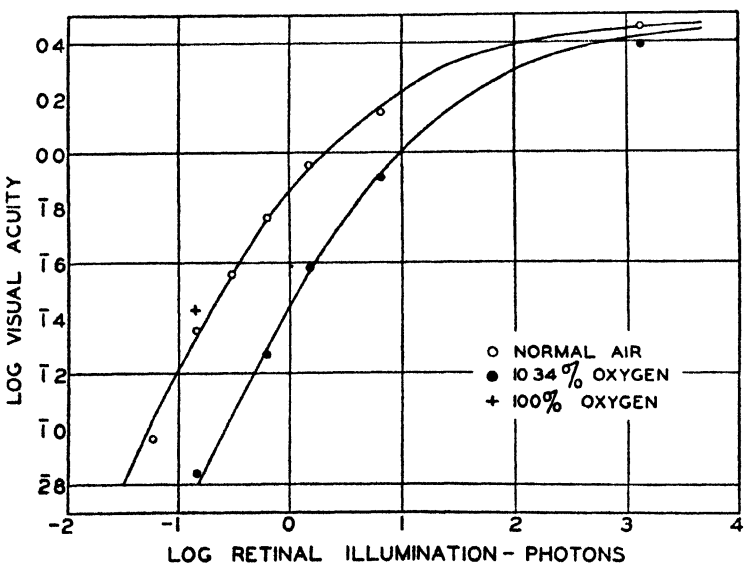

FIG. 2. The data for subject M. O. (Table I). See legend for Fig. 1

the log I axis until a fit for the low oxygen data was obtained. For subject F. B., this shift amounted to 0.38 of a log unit, and for M. O. it was 0.66 of a log unit. In Fig. 3, the same data for subjects M. O. and F. B. have been fitted by the probability integral (*cf.* Crozier, 1937, 1939). Administration of oxygen to each subject at the end of the experiment resulted in complete recovery of visual acuity at an illumination at which it had been most severely affected.

TABLE I

Visual Acuity in Relation to Illumination; Normal Air and 10.34 per cent O_2 (Series 1). (Cf. Figs. 1–3)

Log I in photons	Log visual acuity					
	Subject F. B.			Subject M. O.		
	Normal air	10.34 per cent oxygen	Final control (pure oxygen)	Normal air	10.34 per cent oxygen	Final control (pure oxygen)
$\bar{2}.757$	$\bar{1}.049$	Not measurable		$\bar{2}.961$	Not measurable	
$\bar{1}.159$	$\bar{1}.394$	$\bar{1}.046$	$\bar{1}.460$	$\bar{1}.350$	$\bar{2}.823$	$\bar{1}.431$
$\bar{1}.478$	$\bar{1}.584$	$\bar{1}.355$		$\bar{1}.554$		
$\bar{1}.781$				$\bar{1}.761$	$\bar{1}.268$	
0.166	$\bar{1}.885$	$\bar{1}.726$		$\bar{1}.953$	$\bar{1}.580$	
0.815				0.146	$\bar{1}.911$	
1.147	0.245	0.152				
3.120	0.396	0.375		0.455	0.389	
	Equivalent $\Delta \log I = 0.38$			Equivalent $\Delta \log I = 0.66$		

Series 2.—Table II contains the data for nine subjects breathing normal air, 14.31 per cent oxygen, 10.34 per cent oxygen, and 100 per cent oxygen in that sequence. Each datum of visual acuity represents an average of six to ten measurements. The average values are plotted in Fig. 4. Two points, accurately determined, are sufficient to locate the theoretical curve; this was drawn through the normal air data. It was then translated horizontally to the right so as to coincide with the point representing the measurements at the *low* intensity, at each of the conditions of decreased oxygen tension. The curve thus drawn through the lower point at 10.34 per cent O_2 coincides with the upper point as well. This is confirmed by the fact that the maximum log visual acuity, as calculated from the means of the data in Table II according to Hecht's stationary state equation (*cf.* Discussion) is 0.330 in normal air, and 0.314 in 10.34 per cent oxygen. The standard deviations of the means of log visual acuity upon which the

computations are based (*cf.* Table II) are as high as 0.155, with corresponding standard errors up to 0.052. Consequently the difference of 0.016 between log maximum visual acuity in normal air compared with 10.34 per cent oxygen is negligible, and leads to the conclusion that the translation

TABLE II

Visual Acuity Data for Nine Subjects (Series 2). (Cf. Fig. 4)

Subject No.	Logarithm of visual acuity					
	Dim illumination, log $I = \bar{1}.159$ in photons				Bright illumination, log $I = 3.120$	
	Initial control. Normal air	14.31 per cent O_2 =10,000 ft.	10.34 per cent O_2 =18,000 ft.	Final control. Pure O_2	Initial control. Normal air	10.34 per cent O_2 =18,000 ft.
1	$\bar{1}.386$	$\bar{1}.305$	$\bar{1}.132$	$\bar{1}.460$	0.396	0.375
2	$\bar{1}.412$	$\bar{1}.069$	$\bar{2}.823$	$\bar{1}.431$	0.455	0.389
3	$\bar{1}.296$	$\bar{1}.033$	$\bar{2}.950$	$\bar{1}.255$	0.213	0.125
4	$\bar{1}.086$	$\bar{1}.005$	$\bar{2}.899$	$\bar{1}.151$	0.416	0.379
5	$\bar{1}.221$	$\bar{1}.053$	$\bar{2}.917$	$\bar{1}.221$	0.241	0.207
6	$\bar{1}.314$	$\bar{1}.192$	$\bar{2}.928$	$\bar{1}.389$	0.236	0.225
7	$\bar{1}.336$	$\bar{1}.201$	$\bar{1}.094$	$\bar{1}.417$	0.357	0.375
8	$\bar{1}.408$	$\bar{1}.308$	$\bar{1}.224$	$\bar{1}.495$	0.258	0.249
9	$\bar{1}.349$	$\bar{1}.101$	$\bar{2}.747$	$\bar{1}.355$	0.213	0.219
Mean	$\bar{1}.312$	$\bar{1}.141$	$\bar{2}.968$	$\bar{1}.353$	0.309	0.283
Standard deviation of mean	0.104	0.116	0.155	0.118	0.097	0.098
Corresponding visual acuity, per cent of normal	100	68	45	110	100	94
Difference from normal	—	−0.171	−0.344	+0.041	—	−0.026
Standard deviation of difference	—	0.093	0.158	0.046	—	0.034
Standard error of difference	—	0.031	0.053	0.015	—	0.011
Critical ratio	—	5.52	6.54	2.73	—	2.40
*P	—	Less than 0.01	Less than 0.01	0.03	—	0.05

* P is equivalent to the probability that the observed difference is due to chance. Statistically significant differences are represented by values of P which are 0.05 or less (Fisher, 1932).

of the curve has no appreciable vertical component, under the conditions of our experiments. The maximum visual acuity is not affected by anoxia, although a higher illumination may be required to elicit it.

This consideration justifies our having translated the curves in Figs. 1 and 2 horizontally, although in the case of subject M. O. (Fig. 2) the visual acuity at the highest illumination in anoxia suggests a comparatively slight vertical translation as well. The low value for this one point was probably

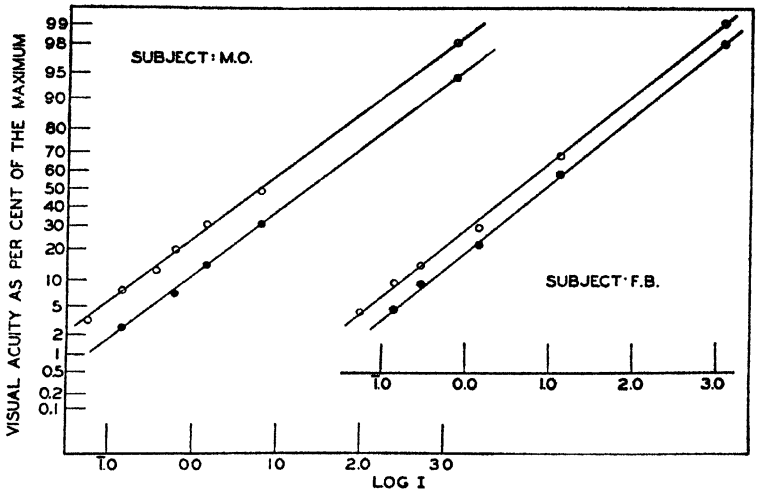

FIG. 3. The data of Table I plotted on a probability integral grid (see Discussion). Visual acuity, as per cent of the estimated maximum, is plotted against the logarithm of retinal illumination in photons. The open circles represent the data in normal air, and the solid circles, those at 10.34 per cent oxygen. The estimated maxima of log visual acuity which were used as parameters are as follows:

	Normal Air	*10.34 per cent O_2*
Subject M. O.	0.461	0.410
Subject F. B.	0.413	0.383

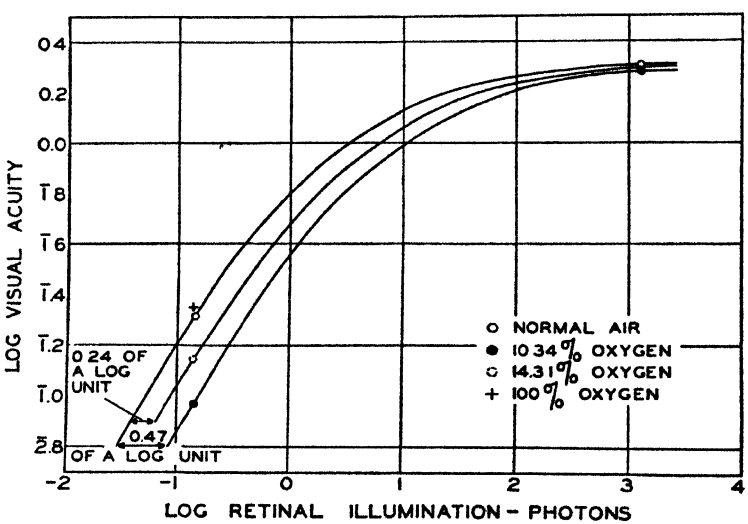

FIG. 4. Mean data for nine subjects, Table II. The curve derived from the stationary state equation (1) has been superimposed on the two points in normal air. It was then shifted *horizontally* until it coincided with the one point at log $I = \bar{1}.159$ for each of the other conditions.

The fact that the curve superimposed in this manner on this point in 10.34 per cent oxygen runs precisely through the point at log $I = 3.120$ as well, leads to the conclusion that there is no appreciable vertical component to the translation which is produced by oxygen deprivation. (See Results, Series 2.)

due to the fact that by the time these measurements were made, the subject was suffering from marked effects of anoxemia, such as headache and vertigo, and it was difficult to obtain his full cooperation. The data for series 2 is more reliable since more determinations at each point were made, and the results for nine subjects are averaged.

The amount of translation of the curve corresponding to the mean values of Table II and estimated from Fig. 4 was 0.24 of a log unit at 14.31 per cent O_2, and 0.47 of a log unit at 10.34 per cent O_2. In Table III are presented the corresponding values for each subject, estimated graphically

TABLE III

Amount of Translation of Curve (Δ log I) for Each Subject of Series 2, Estimated Graphically (As in Fig. 4) from the Data in Table II

Subject No.	Δ log I	
	14.31 per cent O_2	10.34 per cent O_2
1	0.11	0.33
2	0.45	0.75
3	0.37	0.48
4	0.11	0.24
5	0.23	0.40
6	0.17	0.54
7	0.20	0.33
8	0.15	0.27
9	0.36	0.82
Mean	0.24	0.46
Antilog of mean	1.74	2.88
Standard deviation of mean	0.13	0.21

in a similar manner from the data in Table II. The individual differences of the effects due to anoxia were quite large. Their means correspond to the figures estimated from Fig. 3. The amount of translation of each curve on the log I axis is denoted by Δ log I. The antilog of each Δ log I, therefore, represents the factor by which the illumination corresponding to a given level of visual acuity, in normal air, must be multiplied to obtain an equal acuity during oxygen deprivation.

The standard deviations are computed from the formula $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$ where d is the deviation of each datum from the mean, and n represents the number of data. The reliability of the differences in Table II was computed by Fisher's (1932) method for unique samples, and is denoted by P which

represents the probability that the observed difference is due to chance. Values of P of 0.05 or less indicate statistically significant differences.

DISCUSSION

These data may be fitted by a variety of more or less empirical curves. The stationary state equation, for example, has been shown by Shlaer (1937–38) to fit the data of visual acuity, and it is interesting to compare the data obtained in this experiment with the expectation based on the theory of photoreception (Hecht, 1934). Assuming a photosensitive material S which is changed by light to photoproducts P, A, B . . . and which is reformed again by a thermal reaction of some of these products, the equation was derived as

$$KI = \frac{x^n}{(a - x)^m} \tag{1}$$

where I is the light intensity, $(a - x)$ and x are the concentration of sensitive material and photoproducts respectively, m and n are constants giving the orders of the photochemical and thermal reactions, and K is a constant. Shlaer found that in the case of visual acuity mediated by cones, $m = n = 2$, with visual acuity taken as proportional to x^2 and illumination proportional to KI. When these are plotted in the forms of their logarithms, the shape of the curve is independent of the values of K and a which may be assumed, and of the units employed. The value of K merely determines the position of the curve along the intensity axis, while a determines the asymptotic value; the maximum visual acuity corresponds to a^2. The equation may also be written as:

$$KI = \frac{V.A.}{(V.A._{max.}^{\frac{1}{2}} - V.A.^{\frac{1}{2}})^2} \tag{2}$$

These experiments suggest that under reduced oxygen tension the same relationship holds between visual acuity and illumination as under normal oxygen tension, the only change being a shift of the curve to the right along the intensity axis which is equivalent to a decrease in the value of K in the above equation. This means that for any given visual acuity the illumination must be greater at a reduced partial pressure of oxygen than with normal air. One might thus consider the effect of anoxia as being equivalent to placing before the eye of the subject a filter, of a density corresponding to the amount the curve shifts. Subjectively most of the observers reported that the appearance of the field at log $I = \bar{1}.159$ while they breathed 10.34 per cent oxygen resembled that at an illumination 0.4

of a log unit lower during the normal air tests. (This was the reason for giving the subjects of series 2 practice periods at the latter intensity.)

As a consequence of the nature of the relationship between visual acuity and illumination as expressed by the curve in logarithmic coordinates, a shift to the right results in a relatively great decrease in visual acuity at low illuminations. It must be remembered that on a logarithmic scale, equal distances between points represent equal *proportionate* differences. The vertical distance between the curves, which represents the change in logarithm of visual acuity, becomes smaller with increasing illumination and becomes negligible at very high illumination. (This fact explains the inconclusiveness of the previously reported experiments on this problem, since they were performed at a level of illumination at which the changes in visual acuity with anoxia are relatively slight.) The average data of series 2 in our experiments show that at an illumination of 0.144 photons the size of the smallest resolvable detail increased at 14.31 per cent O_2 to 1.48 times that with normal air, and at 10.34 per cent O_2 to 2.21 times normal. At an illumination of 1,320 photons, on the other hand, it increased to only 1.06 times normal, even at 10.34 per cent O_2. It is interesting to note that the behavior of these differences is strikingly uniform for each observer; specifically, the logarithm of the ratio of visual acuity in normal air compared to visual acuity under conditions of reduced oxygen is an inverse function of log I, which approaches zero as I is increased indefinitely.

The figures representing the amount of shift of the curve on the intensity axis are of the same order of magnitude as the shift of the dark adaptation curves upward on the log I axis in anoxia, as reported by McFarland and Evans (1939). The data of the two experiments, however, are not strictly comparable. Several conditions were different in the earlier report. White light was used, yielding data which represent largely the behavior of the rods; no artificial pupil was used; and the observations were binocular.

The results of these experiments and their relation to the data of dark adaptation might be interpreted in accordance with Hecht's (1928) explanation of visual acuity and illumination. Visual acuity depends upon the resolving power of the retina which is composed of discrete rods and cones. Its resolving power is therefore dependent upon the number of functional elements in a unit area. The thresholds of these elements are distributed in a statistical manner similar to that of other populations. As the intensity is increased the total number of elements whose threshold is exceeded also increases, and with it the visual acuity, which is thus determined by the integral form of this distribution curve. At the highest illumination all the cones are functional and no further increase in visual acuity is possible.

The elevation of light threshold during dark adaptation in low oxygen may be considered as indicative of a shift of the distribution curve of the thresholds of the visual elements to the right, toward increasing intensity. This would result in a similar shift of its integral curve—which corresponds to the visual acuity curve—along the intensity axis. That the latter does actually occur is shown by the present experiments. Further, the fact that the maximal visual acuity during anoxia, as computed from (2), is the same as that in normal air would follow from the consideration that under both conditions the maximal visual acuity is determined by the total number of cones per unit area, at least in the emmetropic eye.

The studies of Crozier (1937, 1939, 1940) have demonstrated that various visual data can be fitted at least as well by the probability integral of Gauss as by the stationary state equation. In the case of flicker, when $m = n$ and critical fusion frequency (F) is proportional to the first power of x in equation (1), this equation is mathematically identical with the Verhulst logistic,

$$x = \frac{a}{1 + e^{-p \log KI}}, \quad \text{or} \quad F = \frac{F_{\text{max.}}}{1 + e^{-p \log KI}} \tag{3}$$

in which $p = 1/n$. The shape of this function differs only slightly from the probability integral. In order to determine which fits a set of data more accurately, a large range of very precise data must be available.

In the case of cone visual acuity, which is proportional to the *second* power of x in equation (1), the goodness of fit of the stationary state equation (1) cannot be tested by plotting the data as per cent of $V.A._{\text{max.}}$ on a logistic grid; the relationship is not linear. However, by slightly decreasing the value of the parameter $V.A._{\text{max.}}$ and plotting the data as per cent of estimated maximum visual acuity on a probability integral grid, a rectilinear relationship can be obtained. In Fig. 3 we present the data for subjects M. O. and F. B. treated in this manner. It is to be noted that the fit is certainly as good as with the photochemical (stationary state) equation. The slopes (*i.e.*, $1/\sigma'_{\log I}$) are equal for M. O., although for F. B. a very slight and probably not significant increase of slope in anoxia was noted. In order to obtain a good linear fit for the low oxygen data of both subjects, a lower estimated maximum visual acuity had to be used as parameter than for the normal air data. The evaluation of this parameter in these data is influenced chiefly by the value of one point, that at the highest illumination. As we have already described (*cf.* Results), the mean data of nine subjects (in series 2), which were obtained under conditions which permitted more measurements at each intensity and are consequently more

reliable, contradict such a decrease in maximum visual acuity in low oxygen, although this may be obscured by the averaging process.

The fact that our data can be well fitted by the probability integral is consistent with Crozier's (1940) conception of the determination of visual functions by processes in the central nervous system. Indeed, experiments on the effects of anoxia on the light sense (dark adaptation) suggest that the photochemical system is not affected by anoxia (McFarland and Evans, 1939). Some process further back in the visual mechanism, probably involving the nervous mechanisms of the brain and retina, seems to be altered. However, our data are certainly not inconsistent with Hecht's description of the relationship between visual acuity and illumination under *constant* conditions by means of the photochemical hypothesis.

The changes in visual acuity which were observed in this experiment may be attributed directly to the effects of anoxia for the following reasons. When 100 per cent oxygen was inhaled from a cylinder, through the same breathing equipment but unknown to the subject, normal visual acuity was restored immediately. This occurred at an illumination where the acuity had been severely affected and to a degree which was even somewhat above the acuity while breathing normal air at the beginning of the experiment. Thus, fatigue, the wearing of the mask, and other such factors can be excluded as possible causes of the changes, which were progressive with progressive decrease in oxygen tension. Moreover, although it is well known that anoxia causes psychic disturbances, the changes observed in these experiments are not attributable to a decrease in effort or variation in attention since at the highest illumination, where the test object was smallest and the task consequently most difficult, the changes in visual acuity in each subject were minimal.

The slight improvement of the final control values, compared with the initial tests in normal air, may be attributed to two factors. First, the practice received by the subject in the interval may have resulted in some improvement of his ability to resolve the test object. Second, it is possible that the inhalation of 100 per cent oxygen from a cylinder may result in a slightly higher degree of visual acuity than normal air, although it is an established fact that even under the latter conditions the oxygen saturation of arterial blood is about 95 per cent of its capacity. Rosenthal (1939) has shown that inhalation of 100 per cent oxygen is associated with a contraction of the retinal angioscotomas as compared with normal air, an effect which is in the opposite direction to that caused by anoxia (Evans and McFarland, 1938). This suggests that the slight additional oxygen content of the arterial blood may produce perceptible sensory changes.

As a possible practical application of our results, it might be concluded that, as far as foveal visual acuity is concerned, it is much more important that airplane pilots use oxygen during night flights than during daylight flights. Ferree and Rand (1938) have stressed the importance of a high visual acuity in dim illumination for night flying and night driving.

SUMMARY

1. The foveal visual acuity of eleven subjects was studied in relation to illumination under normal atmospheric conditions and at simulated altitudes of 10,000 feet (14.3 per cent O_2) and 18,000 feet (10.3 per cent O_2). A mask was used to administer the desired mixtures of oxygen and nitrogen. At the end of each experiment, measurements were made while inhaling 100 per cent oxygen from a cylinder. A red filter (No. 70 Wratten) was used so as to study only the behavior of the cones of the retina.

2. The logarithm of illumination was plotted horizontally (abscissa) and the logarithm of visual acuity vertically (ordinate). The reduced oxygen tensions resulted in a shift of the curve to the right, along the intensity axis, the extent of the change being 0.24 of a log unit at 14.3 per cent O_2 and 0.47 of a log unit at 10.3 per cent O_2. These effects were completely counteracted within a few minutes by inhaling oxygen.

3. As a consequence of the shape of the curve, such a shift to the right resulted in a relatively large decrease of visual acuity at low illuminations. At increasing light intensities anoxia produced less and less change, until at very high illuminations the decrease was negligible. Thus with 10.34 per cent O_2 the visual acuity at 0.144 photons decreased an average of 0.344 of a log unit, to 45 per cent of its normal value. At 1320 photons, however, it decreased only 0.026 of a log unit, to 94 per cent of its normal value for that intensity.

The authors are greatly indebted to Dr. Simon Shlaer, of the Laboratory of Biophysics, Columbia University, who kindly placed his visual acuity apparatus at our disposal and made suggestions which were invaluable for the realization of these experiments. We are grateful to Professor W. J. Crozier for suggesting that our data be fitted according to the probability integral function. We also wish to express our indebtedness to The Linde Air Products Company for the generous supplies of nitrogen.

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ADSORPTION OF BACTERIOPHAGE UNDER VARIOUS PHYSIOLOGICAL CONDITIONS OF THE HOST

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INTRODUCTION

The first step in the growth of bacteriophage is the combination of phage with the susceptible bacterial host. The rate of this combination is, under simple conditions, proportional to both the bacterial concentration and to the phage concentration. Various aspects of this process have been studied quantitatively by previous workers (1, 2). Their results will be analyzed and discussed in the sections entitled "Residual free phage," and "Theory of adsorption rates." The main purpose of this paper was the study of a detail of the adsorption process that had not previously received attention, namely the dependence of the rate constant of adsorption on the physiological state of the bacterial host. Such a dependence must be anticipated for two reasons. First, it is known that the size of a bacterium changes very considerably depending on its phase of growth in a given culture medium, and an increased cell surface should lead to an increase of the adsorption rate on to a given number of bacteria. Second, for motile bacteria, like *B. coli*, the adsorption will be faster when the bacteria move about rapidly than when their motility is reduced by adverse physiological conditions.

Our experiments show that the rate constant under optimal conditions is more than sixty times greater than under poor conditions.

Adsorption Rates

The main difficulty in the measurement of adsorption rates is of course the fact that the adsorption process starts off the growth of the phage. Later the phage will be liberated from the bacterial host and will then interfere with the determination of the unadsorbed fraction. For this reason most measurements of adsorption rates have been carried out either

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with dead bacteria or at a temperature sufficiently low to prevent growth. Another method of evading this difficulty consists in making use of the short latent period which elapses between the infection of a bacterium and the release of newly grown phage from it. This method has been used by Schlesinger (reference 2, page 145) and by Ellis and Delbrück (3) and was also used in the present experiments. The limitation in time set by this condition enforces the use of comparatively high bacterial concentrations in order that the decrease of free phage will be measurable. It also requires accurate definition of the time interval to which the measured adsorption is to be referred. This can be achieved by reducing the adsorption rate at the desired moment to one hundredth of its former value by a 100-fold dilution of a test sample.

TABLE I

Adsorption Rate Constants in Cm.³/Min.

Type of bacteria	Physiological state	k cm.³/min.	Observer
B. coli	Live, resting	15×10^{-10}	Schlesinger (2)
"	Heat-killed	6×10^{-10}	"
"	Live, small	15×10^{-10}	Ellis and Delbrück (3)
Staphylococcus aureus	Live, resting	2.2×10^{-10}	Krueger* (1)
"	Heat-killed	2.2×10^{-10}	"

* The numbers in Table IX of Krueger's paper are by a misprint too low by a factor of 100.

We give first a table of adsorption constants as determined by previous observers (Table I).

It will be seen from Table I that the adsorption constants given do not differ very greatly. This is plausible since all the phages used have been selected for their great "activity," so that the adsorption rates are likely to be near the maximum rates attainable, and this maximum rate is determined by the diffusion constant and the size of the adsorbing organism, as will be shown later. Krueger reports no difference between the rates of adsorption on live and on heat-killed bacteria, whereas Schlesinger finds a 2.5-fold greater rate for live than for heat-killed bacteria. This difference may be due to the fact that Krueger's measurements with live bacteria were done at 10°, when they were truly resting, whereas Schlesinger measured at 37°, where the bacteria, though in their lag period and not dividing, were probably actively growing in size.

In Fig. 1 we give some results obtained with our new strains, B_2 and P_2,

of *B. coli* and homologous phage.[1] They show that the free phage falls off exponentially in all cases and that the rate constants derived from the slope

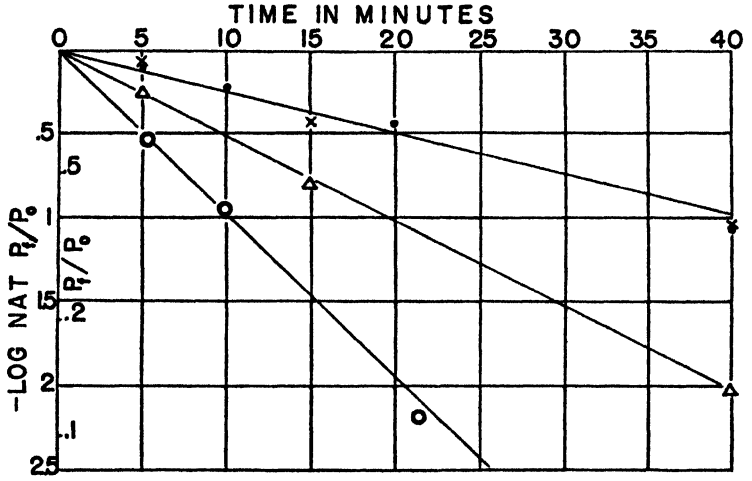

FIG. 1. Adsorption of phage by live bacteria in or near the stationary growth phase, at 25°C.

The bacteria were grown in broth. At time zero they were mixed with the phage. At the intervals given by the experimental points a sample was taken and at once diluted 1:100 in broth to prevent further adsorption. The diluted sample was then in some cases at once, in others at the end of the experiment centrifuged for 4 minutes to throw down the bacteria with those phage that had been adsorbed. A sample from the supernatant was then plated with bacteria. The latent period of phage growth at 25°C. is considerably longer than 40 minutes.

The bacterial concentrations, their cultural conditions, and the adsorption rate constant derived from the experiment were as follows:

	$[B]$ cm.$^{-3}$	k cm.^{3}/min.	Cultural conditions
●	5×10^7	5.4×10^{-10}	Grown with aeration up to a density of 10^9/cc. Diluted 1:20 in broth
×	1.1×10^7	23×10^{-10}	Grown for 30 hrs. without aeration, diluted 1:10 in broth 30 min. before experiment
○	10×10^7	10×10^{-10}	Grown with aeration up to a density of 10^8/cc.
△	10×10^7	5.2×10^{-10}	Grown in broth without aeration for 24 hrs. Diluted 1:2 in broth 10 min. before experiment

of the straight lines of the plot on a logarithmic scale are again similar to those obtained previously on other organisms although there are undoubt-

[1] For a description of these strains see the following paper (*J. Gen. Physiol.*, 1940, **23**, 643).

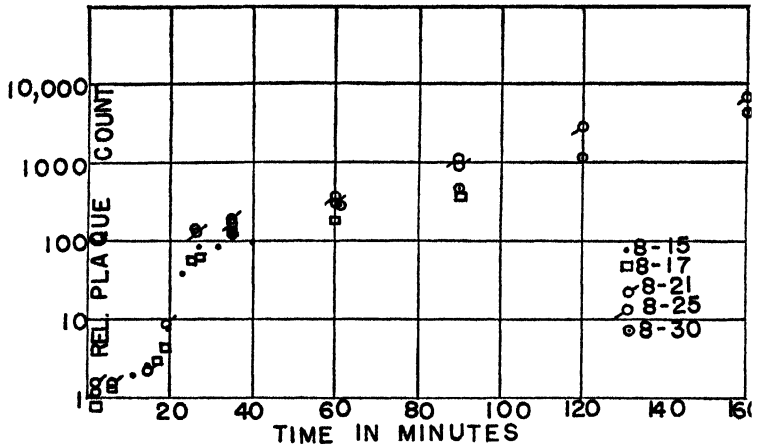

FIG. 2. One step growth in broth at 37°C.

At time zero phage are added to an actively growing aerated broth culture of bacteria, which had been inoculated 3 hours earlier. At time zero it contains about 10^8 *B*/cc. After 5 minutes about 90 per cent of the phage are adsorbed. The mixture is then diluted $1:10^4$ or $1:10^5$ in order to reduce the rate of infection of bacteria by phage set free in the first rise. It is seen that even at this very high dilution the plaque count increases slowly. That this represents true growth is proven by the next two experiments.

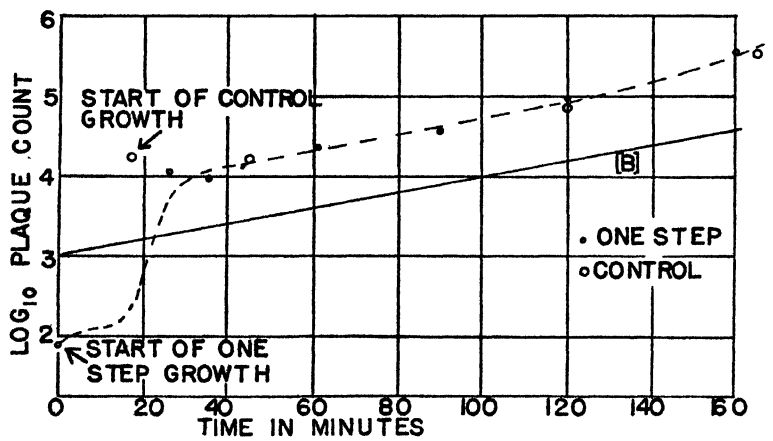

FIG. 3. Proof that true growth of phage occurs at the low bacterial concentrations of the one step experiments.

A one step experiment was carried out with a control, which contained the same concentration of bacteria, cultured under identical conditions and diluted simultaneously with the main culture. But whereas the main culture was mixed with phage 5 minutes *before* the dilution ($1:10^5$), the control was mixed with phage *after* the dilution with such an amount as to make the phage concentration nearly equal to the concentration in the main culture at the end of the step. It is seen that the phage grow equally in both cultures. That this growth proceeds by the normal mechanism, involving, however, an abnormally high adsorption rate is proven by the next experiment.

edly differences between the various batches of bacteria depending on their previous history. Such variations in rate arising from differences in the physiological state will lead to deviations from the simple exponential decrease of the free phage in cases where the physiological state is changing during the course of the adsorption experiment. The failure of some of the earlier observers to establish the simple adsorption mechanism which finds its expression in the straight line dependence is probably due to this complication.

We first obtained an indication that the adsorption rates under optimum physiological conditions might be considerably larger than those measured under the usual conditions in experiments designed to obtain one step growth curves. In these experiments a growth mixture was highly diluted before the occurrence of the first rise in order to prevent reinfection of new bacteria by the phage set free in the first rise. It was found that a 10^4 or 10^5-fold dilution does not suppress further phage increase in the expected degree (Fig. 2). That this increase represents real growth caused by the phage set free in the first rise, and was not due to delayed liberation of phage from bacteria infected before the dilution, was shown by a control in which the phage was added *after* the dilution and to an amount approximating the concentration attained previously by the first step. This control showed the same increase (Fig. 3). We then proceeded to make direct tests of the adsorption rate of the bacteria under these optimal growth conditions, taking points between 1 and 10 minutes. The results are given in Fig. 4 and summarized in the legend. It will be seen that the rate constants are indeed very much higher than in any previous experiments and are of the right magnitude to explain the secondary rise obtained in the one step growth curves after longer intervals.

This result may have some bearing on the activity method of assay invented by Krueger and applied by him and by Northrop in most of their work. In this method the time required to lyse a standard batch of bacteria is taken as a measure of phage concentration. The time interval in question is in part spent by the phage on diffusion preceding the adsorption. It is clear that any slight change in the physiological state of the test sample of the bacteria themselves may greatly influence the adsorption rate and thereby the whole scale of the assay. This is taken care of in Krueger's method by always running a known sample parallel with the unknown in order to establish the scale of the day, but possibly the great day to day fluctuations in this scale are partly caused by this factor.

It should also be noted that by this method adsorbed phage will be assayed higher than free phage, because adsorption of phage is the first stage in the process of lysis. Also the bacterium to which the phage is adsorbed

in the experimental tube will differ physiologically from those used in the assay, and this will alter its growth characteristics and thus its assay value by the activity method.[2] Adsorbed phage and free phage as measured by

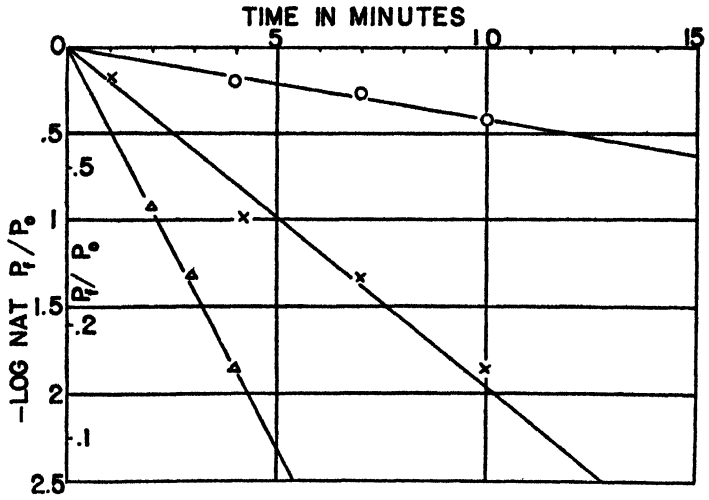

FIG. 4. Adsorption of phage by live bacteria under optimum conditions at 37°C.

The bacteria were grown in broth with aeration at 37°C. They were well in the logarithmic growth phase. Aeration was continued during the adsorption experiment. Technique of determination of free phage as in previous experiment. The bacterial concentrations and the adsorption rate constants derived from the experiments were as follows:

	$[B]$ cm.$^{-3}$	k cm.^{3}/min.
○	1.3×10^6	310×10^{-10}
×	7.7×10^6	210×10^{-10}
△	4.2×10^7	100×10^{-10}

this method are not comparable. Adsorbed phage may simulate increase in phage where there is only a change in the scale of assay.

[2] This is a point which also disturbs the reliability of the plaque count method. Sometimes differing assay values are obtained for free and for adsorbed phage, although the number of infective units has not changed, particularly when the adsorbing bacteria are in a different physiological state from the plating bacteria. Compare the discussion of the "initial rise" in (3). Also the transfer of the infected bacterium from the experimental tube to the agar plate or to the activity assay tube will be accompanied by a change of *milieu*, sometimes favorable, sometimes injurious or even fatal to the progress of phage production in the host. Refinement of technique in this respect will depend upon a closer study of the conditions of phage growth.

Residual Free Phage

If one follows the adsorption of phage over longer times one finds that the rate soon slows down and a few per cent or a fraction of a per cent will always be found unattached. Quantitative studies of this free fraction at equilibrium have been published by Krueger in 1931 (1) and by Schlesinger in 1932 (2), the first working with staphylococcus using the activity assay, the second working with *B. coli* using the plaque count assay. Their results, viewed together allow a more complete interpretation than is given by the authors.

The free fraction becomes of course very large when the phage are more than a hundred times in excess of the bacteria. This is due to saturation of the bacteria with phage and will not concern us here.

But also when the rate P/B is less than one, not all the phage will be adsorbed. We discuss first the experiments with *heat-killed bacteria.*

(*a*) *Krueger's Results.*—In his experiments the ratio P/B varies between 0.4 and 400. They are all done with the same concentration of bacteria. Beginning with small concentrations of phage it is found that the free phage is very nearly a constant fraction of the total phage (about 0.3 per cent). It increases very slowly to about 0.8 per cent when $P/B = 40$, and then very rapidly, on account of the saturation described before. Krueger interpreted these results in terms of a true equilibrium between adsorption and desorption. If this were the case the free fraction should be inversely proportional to the bacterial concentration. No experiments are given to test this point, but one experiment is given as a test whether or not desorption occurred. An adsorption mixture was diluted 1:10 after "equilibrium" had been reached. No desorption was found. Krueger believes that this is due to the extreme slowness of the desorption. It is indeed true that, proceeding on the assumption of a true adsorption equilibrium, the desorption rate would have to be more than a hundred times slower than the adsorption rate, but since also the amount of bound phage is more than a hundred times higher than the free phage a few per cent of desorbed phage would increase the free phage by a few hundred per cent. Indeed a simple calculation, using Krueger's data, shows that in his experiment the free phage should have increased about eightfold. His experiment therefore disproves the assumption of an adsorption-desorption equilibrium.

(*b*) *An Alternative Interpretation Has Been Proposed and Proven by Schlesinger.*—He shows that the free fraction arises from an inhomogeneity among the phage particles themselves. He distinguishes and actually isolates three groups of phage from a fresh lysate. The first and largest group exhibits a uniform and fast adsorption rate and is irreversibly bound. The second fraction is slowly and reversibly bound, the third fraction is not bound at all by heat-killed bacteria.

The last fraction (and part of the second fraction) is that which is found at equilibrium. It is independent of the bacterial concentration when the bacteria are in excess (reference 2, page 140) and it can be isolated and shown to have little affinity to heat-killed bacteria (reference 2, page 144).

The experiments of Krueger and of Schlesinger on free phage in equilibrium with heat-killed bacteria are therefore compatible and can be interpreted with Schlesinger's assumptions of an inhomogeneity among the phage particles.

On the other hand the situation is not so clear with respect to the equilibrium between phage and *live bacteria.*

TABLE II

Test of Affinity of Residual Phage Fractions

	1st Tube 0.4 cc. *B*		2nd Tube 0.4 cc. *B*		3rd Tube 0.4 cc. *B*		4th Tube 0.4 cc. *B*	
Time, *min*.....	0	10	16	26	32	42	48	58
	Add 0.1 cc. stock phage, assay stock phage	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant
$P_f = \frac{P \text{ in supernatant}}{P \text{ added}}$		0.16		0.35		0.36		0 38
Fraction of stock phage-free		0.16		0.05		0.018		0.007

24 hrs. aerated bacteria were used for adsorbing the phage. The free phage in the supernatant of one tube were tested for their affinity to the bacteria in the next tube. 25°C. The experiment shows that the residual phage is qualitatively different from the main bulk.

The difficulty of such measurements is of course the fact that the phage can grow in the presence of living bacteria and that this must either be prevented or taken into account in an unambiguous way.

Schlesinger makes use of the latent period of growth. He leaves the phage in contact with a batch of bacteria for only 10 minutes, then removes these bacteria by centrifugation, and tests the remaining free phage for their affinity to a new batch of bacteria. He finds that the first batch leaves 2 per cent free phage, the second batch leaves 10 per cent free, finally the sixth batch leaves 70 per cent free (reference 2, page 145). This proves that there is a small fraction of phage with little affinity even to live bacteria. We have repeated these experiments with similar results. (See Table II.)

In Krueger's experiments with live bacteria growth of phage was prevented by working at 10°C. He finds that 2 hours after mixing phage and bacteria equilibrium is attained. The free phage is then proportional to the total initial phage (as he and Schlesinger found for heat-killed bacteria), but he also finds that the free phage is inversely proportional to the bacterial concentrations (in contrast to Schlesinger's result with heat-killed bacteria when the free fraction was independent of the bacterial concentration). This would suggest a simple adsorption-desorption equilibrium, a view which is further supported by one experiment, which indicates that desorption occurs at

TABLE III

Desorption Test

0.1 cc. stock phage — 0.9 cc. 24^h aerated *B* (2.1×10^7 plaques/cc.) wait 14 min.	
Centrifuge assay supernatant 1.7×10^6 plaques/cc. P_f = 8 per cent	Dilute 1:100 in supernatant of 24^h aerated *B* (2.1×10^5 plaques/cc.) wait 14 min. Centrifuge assay supernatant 1.8×10^4 plaques/cc. P_f = 8.5 per cent

Phage are left in contact with a thick culture of 24^h aerated bacteria (2.5×10^9/cc.) until adsorption has become slow (14 minutes). A sample is then used for free phage assay, another is diluted 1:100 in the same medium, namely 24^h aerated culture, from which the bacteria have been removed by centrifugation. Free phage is again determined after 14 minutes. No desorption is found.

the required rate. We have found no trace of desorption. (See experiment, Table III.)

Although Krueger's experiments are quite consistent in themselves and could not easily be explained otherwise, we hesitate to accept the interpretation of their author because it requires us to accept that phage can be desorbed from live resting but not measurably from heat-killed bacteria, although the *ad*sorption rates are found to be equal in both cases.

Theory of Adsorption Rates

Schlesinger (reference 2, page 155) has given an interesting theoretical discussion of the adsorption rate by adapting the theory of coagulation of

von Smoluchowski (4) to this case. We will give a simplified presentation of the theory and compare it with some of the experimental results.

Let us calculate the average density distribution around a resting adsorbing sphere of radius a (bacterium) suspended in a medium that contains initially a uniform density of particles which may stick to the sphere when they encounter it. The average density near the sphere will decrease because the particles are constantly withdrawn by adsorption and soon a stationary density gradient will be set up. The density c as a function of r, the distance from the center of the sphere, is then

$$c(r) = c_{\infty}(1 - a'/r)$$

where c_{∞} is the average density at a great distance from the sphere, and a' is a constant which depends on the probability of adsorption once a particle comes close enough to the surface of the sphere for direct interaction. If every approach to the surface leads to adsorption the density near the surface must drop to zero and we must therefore have $a' = a$. If not every approach is successful, the density near the surface will be positive and $a' < a$. This stationary density distribution is quite independent of the diffusion constant. It causes, however, a constant flow F towards the surface which is proportional to the density gradient *and* to the diffusion constant D.

$$F = D \cdot 4\pi r^2 \cdot \frac{\partial c}{\partial r} = c_{\infty} \cdot 4\pi D a'$$

This flow towards the spheres (bacteria) represents the loss of free particles by adsorption. The adsorption rate constant according to this model is therefore

$$k = 4\pi D a'$$

where a' is smaller or equal to the radius a of the adsorbing particle. Since the theory gives only an upper limit for a', it gives also only an upper limit of the adsorption rate, which will be attained when every collision with any part of the bacterial surface leads to adsorption

$$k_{\text{max.}} = 4\pi D a$$

Let us evaluate this upper limit for the staphylococcus phage studied by Northrop and Krueger. The diffusion constant was determined by Northrop (5). He found values between 15×10^{-7} and 0.75×10^{-7} cm.^{2}/ min. The higher value was found at small concentrations of phage, corresponding to those in adsorption experiments. The radius of the coccus we can take to be 5×10^{-5} cm. With these values we obtain as the upper limit of the rate constant $k_{\text{max.}} = 18 \times 10^{-10}$.

This is about eight times greater than the value determined by Krueger (see Table I). The lower value of the diffusion constant would make the observed k greater than the $k_{\text{max.}}$ allowed by the theory and can therefore be ruled out on the basis of this theory.

Our own much higher rate constants for *B. coli* under optimum conditions can be ascribed to two contributing factors. First the considerably larger

surface of the bacteria (perhaps ten times the minimum value). Second, the model underlying the theory is not quite adequate. The *B. coli* will not be stationary under the conditions mentioned but will be actively moving and moreover their range of action is extended through their flagellae. Both the mobility as such and the movement of the flagellae as such will cause the solution near the bacterial surface to circulate rapidly. The average density near the surface will be raised by this circulation to nearly the average density in more distant parts, and the adsorption rate will thus be increased.

Krueger (1) has recorded experiments, in which he varied the viscosity of the suspension by the addition of glycerol. This should, according to Einstein's formula, alter the diffusion constant and according to the above theory cause a proportionate change in the adsorption rate. This conclusion is quite independent of the type of reaction that takes place at the bacterial surface itself. Krueger's experiments do not show this expected dependence of the adsorption rate on the viscosity. This seems quite inexplicable.

DISCUSSION

The study of the adsorption of phage onto its sensitive host is a study of the diffusion of phage through the medium and of its specific attachment to a host. This attachment has many analogies to the antigen-antibody reaction (Burnet). The whole process of adsorption is a necessary forerunner of the multiplication of phage under ordinary conditions. In itself it has nothing to do with the growth process, but in non-lysogenic strains it is intercalated between the true growth reactions and necessitates a careful separate study of the adsorption before one can draw relevant inferences from growth experiments. The adsorption interferes in two ways. First in the experimental vessel. Here the fact, that the adsorption proceeds continuously, has the effect of smoothing out the steps that arise from the sudden liberation of new phage from infected bacteria. A judicious adjustment of the bacterial concentrations, so as to make adsorption either "infinitely fast" or "infinitely slow" in comparison to the latent growth in the bacteria, is therefore necessary to bring out the steps described by d'Herelle, by Ellis and Delbrück (3), and in the following paper by Delbrück (6).

Second, it interferes in the assay of phage, whether the plaque count or the activity method is used. The effect on the plaque count is small, and this can probably be understood by the following argument. In order that a phage particle may form a plaque it must infect a living bacterium *before* the bacterial layer has grown so thick, that growth conditions for phage

become unfavorable. In general there will be ample time for the majority of phage to fulfill this condition even if the bacteria are of the poorly adsorbing variety. Adsorption rate is therefore not a limiting factor for the efficiency of plating. This is borne out by the observation, that the efficiency of plating is only slightly changed if rapidly growing bacteria are used for plating instead of the standard 24 hour aerated cultures.

The converse is true for the activity method. Here the time spent by the phage particles on adsorption is an integral part of the quantity measured, namely the time between the addition of phage and lysis, and any factor that changes the adsorption rate (or eliminates it, as in the assay of adsorbed phage) will shift the scale of the assay. As we have shown, the adsorption rate does in fact change with the physiological state of the bacteria, and it is different for different fractions of the phage. In fact, if Schlesinger's discovery holds true also for staphylococcus phage, it would appear that the activity method was ill-suited for the quantitative determination of the residual free phage, since the experiment selects those particles whose assay value is least comparable with those of the main bulk.

SUMMARY

1. The adsorption rate constant of phage to bacterium is found to change between wide limits, depending on the physiological state of the bacterium.

2. The experiments of Krueger and of Schlesinger on the residual free phage in contact with an excess of bacteria are discussed and the view of Schlesinger, that they represent phage particles with reduced affinity to the bacterial host is supported by experiments.

3. The theory of von Smoluchowski and Schlesinger is compared with the experiments.

4. The implications of these findings for the assay methods currently used are discussed.

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THE GROWTH OF BACTERIOPHAGE AND LYSIS OF THE HOST

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Introduction and Statement of Main Result

Bacteriophage grows in the presence of living susceptible bacteria. In many but not all cases the growth of phage leads finally to a lysis of the bacterial cells, a phenomenon which in dense cultures manifests itself to the naked eye as a clearing of the bacterial culture. The exact nature of the connection between the growth of the phage and the dissolution of the cells has been a subject of controversy since the original discoveries of d'Herelle in 1917.

D'Herelle believed that lysis is the process by which the phage, which has grown within the bacterium, is liberated from the cell and dispersed in solution. Many later authors, notably Burnet, have concurred with him on this point. Last year Ellis and Delbrück (1) published detailed evidence that phage liberation in *B. coli* occurs in sudden bursts and showed that all the evidence was compatible with the assumption that in sensitive strains the bursts of phage liberation occurred only if and when a cell is lysed.

Northrop and Krueger (3–5) on the other hand have developed ideas along a somewhat different line in the course of their extensive research with a strain of *Staphylococcus aureus* and a bacteriophage active against it. Bordet (2) had put forward the conception that phage production followed by lysis is a more or less normal physiological function of the bacteria. In lysogenic strains where visible lysis never occurs it can be put into close analogy with the production of an extra-cellular enzyme. Northrop's and Krueger's work served to substantiate this view also in their case where the phage growth leads finally to the dissolution of the bacteria. In their view lysis of the bacteria is a secondary and incidental activity of the phage.

Krueger and Northrop (3) found first that clearing, if it occurs at all, begins when a certain threshold value in the ratio total phage/bacteria is

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overstepped. A considerable *loss in total phage* parallels the clearing of the culture. If sufficient phage is added so that the ratio phage/bacteria is greater than the threshold value lysis begins almost at once. This experiment was later repeated and confirmed by Northrop (4, 5) with purified concentrates of phage.

Recently Northrop (5) found with a susceptible *megatherium* strain and homologous phage that the bulk of the phage was liberated *before* the culture began to clear. He found further with a lysogenic strain which never showed clearing but produced phage lysing the sensitive strain, that the yield in phage from this lysogenic strain was large compared to the number of bacteria present in the culture.

All these results indicate that in these strains lysis, if it occurs at all, is brought about by a mass attack of the phage on the bacteria *after* the phage have grown and been liberated into solution.

We have now studied in more detail the relation between phage growth and lysis in a new sensitive strain of *B. coli* and homologous phage and have obtained results which may offer a basis for reconciling the two diverging lines of interpretation.

We have found in this strain two entirely different types of lysis, which we designate as "Lysis from within" and "Lysis from without."

Lysis from without is brought about almost instantly by adsorption of phage at a threshold limit, which is equal to the adsorption capacity of that bacterium. No phage are liberated in this case, on the contrary, the adsorbed phage are lost. The phage attack the cell wall in such a way as to permit swelling of the cell, and its deformation into a spherical body.

Lysis from within is brought about by adsorption of *one* (or few) phage particle(s). Under favorable conditions this one phage particle multiplies during a latent period within the bacterium up to a threshold value (which is equal to the adsorption capacity). When the threshold value is reached, and not before, the phage is liberated by a sudden destruction of the protoplasmic membrane, which permits a rapid exudation of the cell contents without deformation of the cell wall.

It would seem that the results of other observers may be explained by postulating that

(*a*) In the case of *Staphylococcus aureus* the observable clearing is caused by lysis from without. Lysis from within either does not exist here and is replaced by continuous phage secretion; or it exists but leads only to a slow equalization of the refractive indices of the cell interior and the *milieu*. The decrease of total phage during lysis is caused by the adsorption of phage in the process of lysis from without.

(*b*) In the case of *B. megatherium* 36, sensitive, (Northrop (5)) the same relations hold.

(*c*) In the case of *B. megatherium* 899, lysogenic, (Northrop (5)), lysis from without does not occur, although the bacteria can adsorb a few phage particles each. Both the phage production capacity and the phage adsorption capacity are far smaller than the corresponding value for the same phage acting on the sensitive strain.

The equation

$$\text{Adsorption capacity} = \text{maximum yield of phage per bacterium}$$

was found to hold true both for bacteria in the phase of rapid growth, and for saturated bacterial cultures that had been aerated for 24 hours and consisted only of very small bacteria.

This equality points to a material connection between the bacterial constituents which can adsorb the phage and the new phage formed when it grows. These bacterial constituents we shall call *b*. It might be assumed that *b*, which the bacterium constantly produces without the help of phage (and in some cases also secretes), is part of the precursor which under favorable conditions is transformed into phage after combination of the bacterium with a phage particle from without. The complex *bP* might be the catalyst which *in the cell* transforms uncombined *b* into phage. The difference between a sensitive strain and a lysogenic strain would consist in this: in the sensitive strain the reaction

$$b \xrightarrow{\text{catalyzed by } bP} \text{phage (in the cell)}$$

would be faster than the production of *b* (in the cell). In the lysogenic strain *b* would be produced faster than it is converted into phage. This permits the bacterium *and* the phage to grow.

The extremely interesting but puzzling observations of Burnet and McKie (6) on lysogenesis of different variants of one strain of *B. enteriditis* Gaertner, and of Burnet and Lush (7) on induction of resistance and lysogenesis by the phage in a strain of *Staphylococcus albus* may perhaps allow further analysis in the light of these speculations.

EXPERIMENTAL

The strains of B. coli and of homologous phage used in this work were obtained from the Pasadena Junior College, through the courtesy of Mr. F. Gardner. They have not been studied before and will be designated as B_2 and P_2, in distinction to the strains B_1 and P_1 used last year by Ellis and Delbrück (1).

Growth curves of B_2 in Difco nutrient broth at 37° (by colony counts) are shown in Fig. 1. The maximum division rate is considerably smaller than that of B_1.

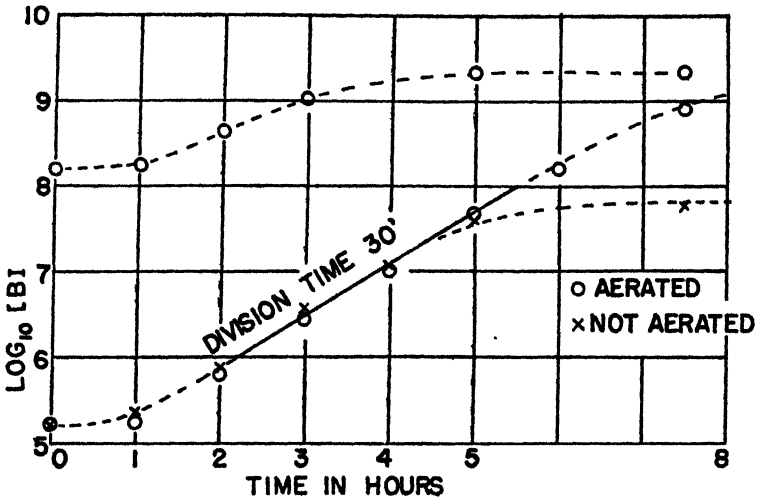

FIG. 1. Growth of bacteria in broth at 37°.

The inoculating bacteria were taken from an 18 hour not aerated broth culture. Without aeration the growth reaches saturation at 10^8 B/cc. With aeration the growth proceeds further beyond 10^9 B/cc. The maximum growth rate is in both cases equal and corresponds to an average division time of 30 minutes. The cessation of growth in the unaerated culture is therefore caused by lack of air. This is further supported by the top curve, which shows how the unaerated 18 hour culture, without the addition of fresh broth, proceeds to grow, when aerated, and reaches about 2×10^9 B/cc.

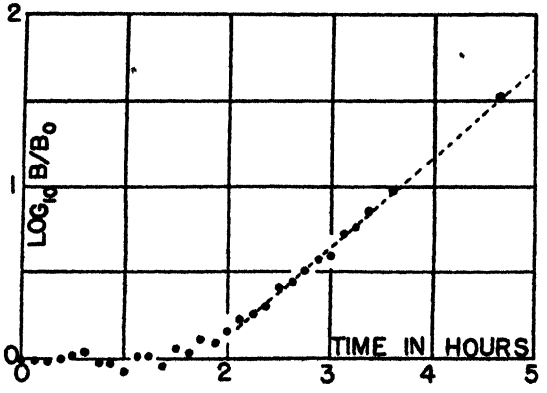

FIG. 2. Growth of the standard experimental culture of bacteria in broth at 37°.

At time zero 0.1 cc. of a 24 hour stock culture was added to 25 cc. broth. Every 7.5 minutes platings were made for colony counts. The plotted values show complete agreement with Hershey's (11) finding that such aerated bacteria exhibit a sudden transition from the phase of cell enlargement (lag period) to the phase of cell division. The lag period is 2 hours, if defined as the time required for increase in cell number by a factor 1.5 (compare Hershey's discussion (11)).

Synthetic Medium.—The bacteria and phage were also grown with aeration in a synthetic medium, consisting of

l-Asparagine	2 gm.
Glucose	4 gm.
Na_2HPO_4 (anhydrous)	6 gm.
KH_2PO_4	3 gm.
$MgSO_4$	0.05 gm.
NaCl	0.05 gm.
Distilled H_2O	1000 cc.

The bacteria in this medium grow more slowly than in broth but attain a higher final concentration. The phage also grow well on bacteria in this medium and cause lysis. But the growth rates of both the bacteria and the phage are only approximately reproducible with different batches of medium. These irregularities must be eliminated before the medium can be used for quantitative studies.

Bacteria transferred from this medium into broth grow at once. Transferred from broth to this medium they require a period of about 24 hours of adaptation before growth begins.

The bacteria were therefore carried on slants of synthetic medium agar and transferred into broth only for the specific experiments.

Stock phage was obtained by lysis in broth and filtration through Jena sintered glass filters. No measurable decrease in titer in periods over 6 months (in contrast to P_1, compare (1)).

Phage assay by plaque count on Difco nutrient agar plates, as described previously (1). The plaques are large (2 mm. diameter) and are countable after 4 hours incubation at 37°.

Turbidity was determined by visual comparison with turbidity standards, in most cases taken from the same culture. Such turbidity determinations are of course very rough, but a refinement of technique in this respect did not seem profitable. The turbidity in any event would not be proportional to the lysis, which in dense cultures, as we have seen, is a complex phenomenon, in which changes in shape, size, and refractive index occur. Each of these factors contributes to the turbidity change.

Preparation of Phage Concentrates.—For the experiments with large excess of phage over bacteria stock phage of very high titer were needed. These were obtained in the following way.

It was observed that lysates obtained from *synthetic medium* cultures (in contrast to broth lysates) lost all their phage on filtration through Jena sintered glass filters. The phage is not inactivated by the filter but simply adsorbed, and it can be eluted with good yields by small volumes of distilled water. An example is given in Table I.

These concentrates could be further concentrated by adding phosphate buffer to restore the original salt concentration and repeating the adsorption-elution procedure.

Filters of the coarser grade 4 were also tried and though effective gave less reliable yields.

Ground glass, silica, and fullers' earth were tried as adsorbents. These also gave good adsorption but the elution was again often unsatisfactory.

The phosphate buffer was replaced by 1 per cent $MgCl_2$ solution and by a 1 per cent

NaCl solution. Both were as effective as phosphates in causing the phage to be adsorbed by the filter.

Finally various concentrations of phosphate buffer were tried in order to determine the lower limit at which adsorption would take place. It was found that reduction of the buffer concentration to one half practically eliminated the adsorption.

By this method several concentrates were obtained with titers between 10^{11}/cc. and 10^{12}/cc. These concentrates were clear in transmitted light and showed intense blue Tyndall scattering. When kept in the ice box they showed no measurable decrease of titer over periods of more than 5 months.

Standard Cultural Conditions.—The cultural conditions of the bacteria were standardized in the following way. A stock culture was kept going for about a week by daily transfers into fresh synthetic medium. At the time the transfer was made, the culture contained about 2.5×10^9 bacteria/cc. These 24 hour aerated bacteria were used for phage assay. They will be referred to as the *stock culture.*

TABLE I

Adsorption and Elution of Phage from Jena Sintered Glass Filters

	Assay	Total phage
25 cc. fresh lysate	1.4×10^{10}	35×10^{10}
Filter lysate through Jena sintered glass filter grade 5 on 3, filtrate	0.001×10^{10}	0.025×10^{10}
Follow with 2 cc. distilled water	6.4×10^{10}	12.8×10^{10}
Follow with 5 cc. distilled water	0.19×10^{10}	0.95×10^{10}

At the time of the transfer of the stock culture a sample of 0.1 cc. was also transferred into 25 cc. *broth* and aerated at 37°. This culture was used for growing the phage, and will be referred to as the *experimental culture.*

Fig. 2 shows the growth of this experimental culture as determined by colony counts in 7.5 minute intervals. It is seen that the bacteria do not start to divide in the first 90 minutes and divide at maximum rate after 3 hours. It can therefore be used to study phage growth either on bacteria that are small and have a long period of growth in size without divisions ahead of them, or on bacteria that have attained their maximum average size and divide at a maximum rate.

Microscopic Observations

We have made some microscopic studies of the lysis of our bacteria under various conditions, both in hanging drop preparations and on nutrient agar plates. The hanging drop preparations have the advantage that one can study changes in mobility of the bacteria and also that the bacteria are subjected to more uniform conditions, while on the agar plate one can follow the history of individual bacteria over a stretch of time. These observations were made at room temperature, the time schedule is therefore retarded in comparison with the growth curves at 37°C.

Great differences in behavior were found depending on whether the bac-

teria were infected with about an equal number of phage or with a large excess of phage (200 to 1 or still higher ratios) and these will be described separately.

1. $P/B = 1$

(*a*) *Hanging Drop*

No changes in the shape of the bacteria were observed, only a gradual diminution in the number of bacteria.

(*b*) *On Nutrient Agar Plates*

The bacteria were first mixed with phage in broth and aerated at 37° for 10 minutes, as in an ordinary growth curve set up. At 10 minutes a 0.1 cc. sample was spread on an agar plate and observed under the microscope (magnification 600). A map was drawn of about 100 bacteria in the field of vision and these were checked every 5 minutes for changes. Up to about 30 minutes (now at room temperature, 25°) only a few bacteria disappeared, the others showed no change. Between 30 and 70 minutes 70 per cent of the bacteria disappeared. They disappeared by a process of fading out, *without noticeable change in shape.* The fading out takes about 2 minutes, a faint outline of the rod remaining visible for a long time afterwards. There appeared to be no correlation between the size of the bacterium and the inception of its fading. Many bacteria which at the beginning had a constriction as if they were on the point of dividing behaved as one bacterium on lysis. Sometimes the fading appeared to start at one end of the rod and then to proceed gradually through its entire length.

Discussion.—The bacteria are visible without staining under the microscope by virtue of a difference in the refractive index between their interior and the surrounding medium. The fading out without change of shape means then that the refractive index of its contents becomes equal to that of its *milieu*, while the cell wall retains its form. Inside and outside must suddenly become capable of free exchange. This must be due to a sudden disruption of the protoplasmic membrane. A change in permeability could hardly be so drastic as to permit complete equalization in so short a time.

2. $P/B = 200$ or greater

(*a*) *Hanging Drop*

The bacteria kept their normal size and rod shape up to about 20 minutes. Then suddenly within 1 or 2 minutes the large majority was transformed into spherical bodies of about the same volume and small refractive power. These spherical bodies were visible for a long time and only gradually decreased in number, some could be seen much distended and of oval shape.

Besides these spherical bodies there appeared a few very minute rods that were extremely motile.

Also some of the spherical bodies showed great motility.

(*b*) *On Nutrient Agar Plates*

The bacteria were mixed with a 200-fold excess of phage in broth for 5 minutes to permit time for adsorption. Then a 0.1 cc. sample was plated and observation began at 10 minutes. No changes in size or shape were observable up to 18 minutes. Then first few and soon many of the bacteria exhibited a variety of changes in form with parallel slow fading out. In most cases the rod simply swelled to an oval or spherical shape. Sometimes the swelling began at one end giving the impression of a rod attached to a little sphere, gradually the rod shortened and the sphere grew until only the sphere was left, which later assumed an irregular shape and finally faded out. Often the swelling began in the middle at the constriction of a dividing cell

TABLE II

	No. of bacteria lysed by	
P/B	Fading	Swelling
1.3	83	0
1	66	0
1	59	0
25	43	9
150	3	15
250	0	12
300	1	43

and then extended to both ends until only one sphere was visible. The whole process from the inception of swelling to the attainment of a spherical shape took between 2 and 10 minutes. The spheres then fade out very slowly.

Also a few minute rods are seen on the agar plate. They are never lysed. Their genesis has not been observed.

Discussion.—It is clear from the above description that lysis under the influence of many external phage particles is an entirely different phenomenon from lysis under influence of one external phage particle which has grown within the bacterium to a large number. We must distinguish between lysis from within and lysis from without. In the latter case apparently the phage *en masse* attack the cell wall and so alter its elastic properties as to permit swelling of the cell and the uptake of water. Possibly the cell wall is actually dissolved and only the protoplasm remains and swells up freely.

3. P/B between 1 and 200

For these intermediate cases observations on agar plates showed not an intermediate type of lysis but a gradual shift of the *fractions* of bacteria that are lysed by fading or by swelling respectively. Table II illustrates this point.

There is no ambiguity regarding the type of lysis a particular bacterium has undergone, except in rare cases when the fading has proceeded too far by the time of the next inspection, so that the form cannot be ascertained any more.

Bronfenbrenner, Muckenfuss, and Hetler in 1927 (12) and Bayne-Jones and Sandholzer in 1933 (13) have published very interesting photomicrographic moving pictures of lysing bacteria. They describe essentially the same morphological types of lysis which we find. In their experiments, however, the conditions of infection were not systematically varied and the ratio phage/bacteria was in no case determined. The significance of variations in the lytic process was therefore not recognized.

One Step Growth Curves

Phage growth curves with these strains of phage and bacteria show the same general features as those described by Ellis and Delbrück (1) for the strains B_1 and P_1. If phage is added at time zero to an excess of bacteria the plaque count stays constant for 17 minutes, then rises, at first sharply and then more gently (on the logarithmic plot!) until about 30 minutes. At that time the first step is nearly completed. If the growth mixture has not been diluted before the beginning of this first rise another sharp rise begins at about 34 minutes (Fig. 3). If reinfection has been prevented by extreme dilution there is very little further increase in plaque count (see Fig. 2 of the preceding paper).

It was decided to study in more detail this first step. The condition of extreme dilution under which the one step growth curve has to be measured facilitates an accurate analysis, because the samples which have to be plated at definite intervals need only be mixed with bacteria and plated, without further dilution. The time to which the assay is to be referred can then be defined within a fraction of a minute.

Fig. 4 shows the values obtained from three such growth curves. It should be noted that since the plaque count is plotted directly (instead of logarithmically as in most plots of this kind) the sampling error (which is proportional to the measured value) is more conspicuous near the upper end of the growth curve. The actual percentage deviations are experimentally larger near the beginning of the rise, because here the plaque

count increases by a factor of twenty in 3 minutes and very slight inaccuracies in timing will entail huge percentage deviations in the plaque count. During the process of plating a bacterium may liberate the phage which it contains and thus add the full number of a "burst" to the normal sample value. This will place the point too high; it was probably the case in the 17 minute point of the first growth curve.

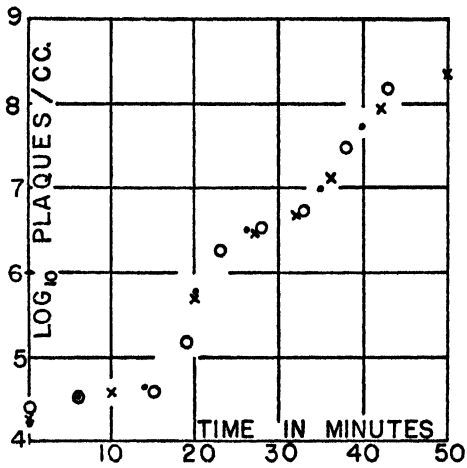

FIG. 3. Phage growth on rapidly dividing bacteria.

At time zero phage were added to the experimental culture of bacteria in its optimal growth phase (3 hours after inoculation) and well below its maximum concentration of bacteria (5×10^7 against 2.5×10^9). The initial phage concentration was 2×10^4, so that even after the first step the bacteria were still much in excess and no multiple infection occurred. After the second step the phage were in excess. The course of events resulting from this situation will be discussed separately.

Dates of experiments: ● 9-13, ○ 9-15, × 9-22.

Fig. 4 brings out very clearly one point which was not recognizable in the logarithmic plots: Phage liberation starts suddenly after the latent period of 17 minutes and continues *at a constant rate* for about 16 minutes, at which point it ceases almost equally abruptly. In this interval from 17 to 33 minutes the plaque count increases by a factor 170.

These characteristics of phage growth, namely the latent period, the spread of the latent period, and the step size depend on the physiological state of the bacteria. For example, if, instead of using the above defined experimental culture after 3 hours when the bacteria are large and divide rapidly we had taken the bacteria from the stock culture directly, the one step phage growth curve under the same conditions (in broth at 37°C.)

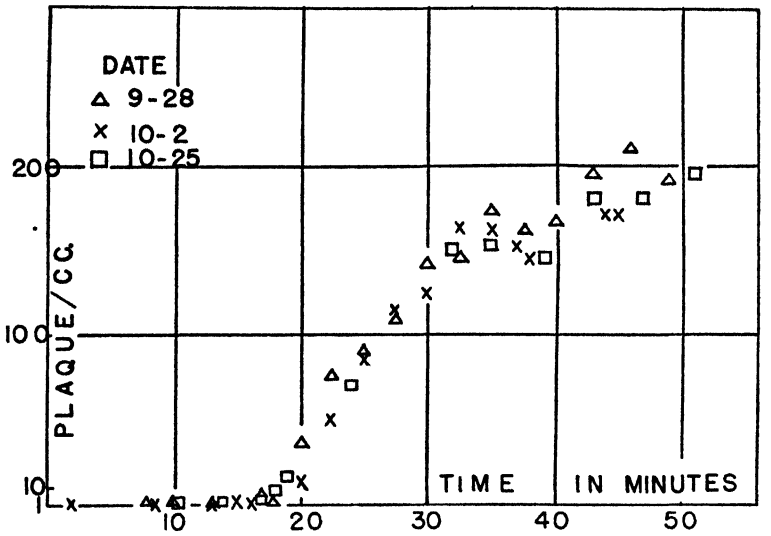

FIG. 4. One step growth curve of phage on rapidly dividing bacteria at 37°. Direct plot.

At time zero about 10^7 phage/cc. were added to 25 cc. of a rapidly growing broth culture of bacteria, that had been aerated for 3 hours and contained about 5×10^7 bacteria/cc. After 5 minutes, 10^3-, 10^4-, and 10^5-fold dilutions of this growth mixture were made in broth of 37° and these were further aerated and incubated. At 1 or 2 minute intervals samples from these mixtures were plated for plaque counts.

It is seen that the plaque count stays constant for 17 minutes, then increases *linearly* with time till 33 minutes when it reaches 170 times the original value. After 33 minutes it stays nearly constant. Phage liberation takes place uniformly during 16 minutes.

It should be noted that on a logarithmic plot the rise would appear to be much more sudden. In fact on such a plot more than half of the step would be accomplished within 3 minutes, when the plaque count has risen to twenty times the original value.

TABLE III

Characteristics of Phage Growth on Rapidly Dividing Bacteria and on 24 Hour Aerated Bacteria, Both Measured in Broth at 37°C., with Aeration

	Minimum latent period	Spread of latent period	Step size	Saturation value
	min.	*min.*		
Rapidly dividing bacteria	17	16	170	250
24 hrs. aerated bacteria	30	22	20	20

would have been qualitatively the same but quantitatively quite different (see Fig. 5). Table III lists the respective values.

The constant rate of phage liberation in the best one step growth curves

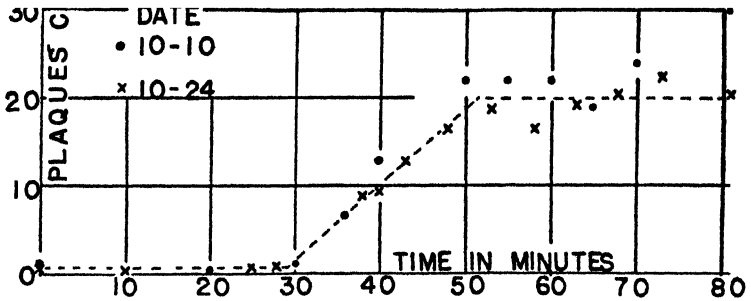

FIG. 5. One step phage growth on 24 hour aerated bacteria. Direct plot.

At time zero 2×10^8 phage/cc. were added to 1 cc. of a 24 hour stock culture of bacteria, containing 2×10^9 bacteria/cc. After 5 minutes free phage were determined and suitable high dilutions in broth were further incubated at 37° with aeration. At intervals samples from these mixtures were plated for plaque count.

It is seen that the plaque count stays constant for 30 minutes, then increases linearly with time till about 50 minutes when it reaches twenty times the original value. After 50 minutes it stays nearly constant. Phage liberation takes place uniformly during 20 minutes.

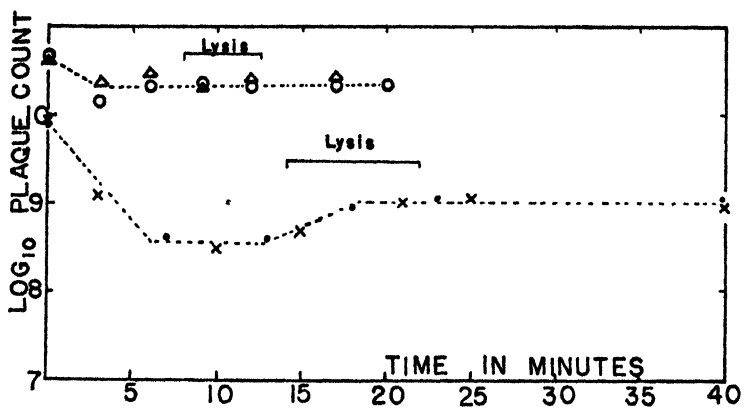

FIG. 6. Addition of a great excess of phage to a growing culture of bacteria, at 37°

	Date	P/B	log P/B	P bound/B	Free phage after adsorption
					per cent
●	9–13	120	2.08	115	4
×	9–22	60	1.78	57	5
○	10–26	500	2.70	250	50
△	11–20	700	2.85	270	60

(Fig. 4) permits a closer analysis. Since the liberation of phage from the *individual bacterium* probably occurs quite suddenly when the bacterium is lysed our result means that the infected bacteria represent a mixture of groups with latent periods ranging between 17 and 33 minutes and that there is a uniform distribution of bacteria over this whole range of latent periods.

The question arises as to what causes a bacterium to have a shorter or longer latent period. Several hypotheses might be suggested, either by ascribing the cause to statistical fluctuations of reactions involving a small number of particles (10), or by connecting it in one way or another with the bacterial cycle. The latter view seems to the author the more likely one but since it has not yet been worked out, further discussion will be deferred.

Multiple Infection

It was reported by Ellis and Delbrück (1) that if a bacterial suspension is infected with an excess of phage no changes occur in the latent period or in the burst size. At that time no phage concentrates were available and the maximum ratio of phage to bacteria attained in that work was only four to one.

We have repeated this work with our new strains and with the concentrates and have been able to work with much higher ratios of phage/bacteria, up to 700 to 1.

Fig. 6 shows some of the results obtained with the high ratios. Samples were assayed every 3 or 5 minutes. Since the assays here require several dilution steps these growth curves are less accurate both with respect to assay values and with respect to timing.

The results show an initial decrease in plaque count because many phage particles are bound to one bacterium which then gives only one plaque. For instance, starting with 10^{10} phage/cc. and a hundred times less bacteria, one finds initially 10^{10} plaques/cc. After 10 minutes only 5 per cent of the phage will be left free; these will give 5×10^8 plaques/cc. In addition the 10^8 bacteria/cc., each having adsorbed on the average 95 phage particles, will give 10^8 plaques/cc., bringing the total plaques to 6×10^8/cc. If the initial ratio phage/bacteria is greater than a certain critical value the bacteria show saturation. This saturation value depends on the physiological state of the bacteria. For instance, for rapidly growing bacteria, if the ratio is 500, the plaque count decreases only by a factor two. The saturation value is therefore 250. On the other hand, for 24 hour aerated bacteria, the saturation value is only about 20. (See Table III, last column.)

We have indicated in the figure the time during which clearing of the mix-

ture occurs. If the initial ratio of phage/bacteria is smaller than the saturation value, clearing occurs only slightly earlier than in a one to one mixture, and it is accompanied by a noticeable increase in the plaque count.

On the other hand if the initial ratio of phage/bacteria is greater than the saturation value, clearing occurs much earlier and is not accompanied by an increase in plaque count.

In both cases the final plaque count is considerably smaller than the initial one; we have, in effect, a phage destruction by the adsorption that causes lysis.

One can see the difference between the two types of lysis with the naked eye. A culture of rod shaped bacteria, like *B. coli*, shows flow lines on shaking due to the orientation of the rods under the influence of the shearing forces of unequal flow. In lyses under the influence of great excess of phage these flow lines disappear before the culture clears up, because the rods are transformed into spherical bodies before they disappear, as described in the section on microscopic observations.

Growth of Phage and Lysis of Bacteria When Equivalent Numbers Are Mixed

It can be predicted that a disturbance must arise when equivalent amounts of phage and bacteria are mixed, due to the fact that the phage that are liberated from the first lysing bacteria will cause an excess of phage over bacteria to be present. These phage will be adsorbed on bacteria that are already infected and will therefore not show up in a plaque count assay. They will moreover interfere with the phage growth in these bacteria and in some of them cause a lysis from without.

Qualitatively the following can be predicted. We have seen that the phage will be liberated at a constant rate (after the lapse of the minimum latent period of 17 minutes). They will be adsorbed at a rate that is proportional to the phage concentration and to the bacterial concentration. The phage concentration is constantly increasing and the bacterial concentration is constantly decreasing (due to lysis). The adsorption rate will therefore pass through a maximum and the net free phage production rate will pass through a minimum. The net result is the appearance of a point of inflection, *i.e.* a secondary step in the phage growth curve, in some cases even a temporary decrease in the free phage if the rate of adsorption at any time exceeds the rate of phage liberation. Because of the loss of phage by adsorption and partial lysis from without the total step size must be smaller than in a one step growth curve where the bacteria are in excess and where multiple adsorption is prevented by extreme dilution, after adsorption of the parent phage.

These predictions are borne out by the experimental results. Fig. 7 shows three such growth curves where nearly equivalent amounts of phage and bacteria were mixed at time zero. The diminished yield is very pronounced and the secondary step is discernible in two sets of observational points. The condition of single infection of all bacteria at zero time can of course be realized only approximately. Even if exactly equivalent amounts

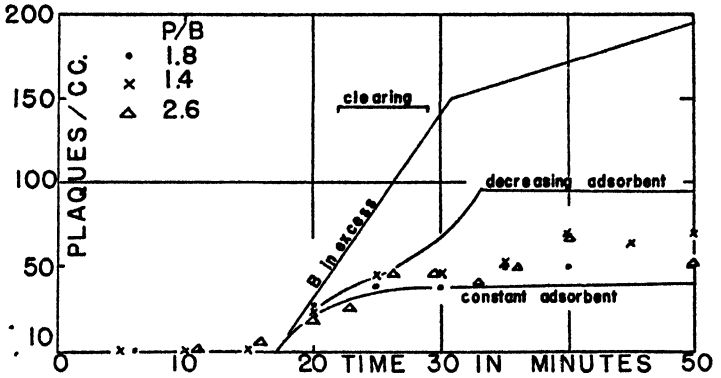

FIG. 7. Growth of phage if equivalent high concentrations ($\sim 10^8$/cc.) of phage and bacteria are mixed at time zero. Direct plot.

Besides the experimental points from three growth curves three theoretical curves are drawn in the figure. These are

1. A one step growth curve with B in excess, taken from Fig. 4.
2. A calculated growth curve, assuming inactivation of the liberated phage on bacteria not yet lysed.
3. Same, but assuming that the adsorbing power of the bacterial constituents responsible for it is unimpaired till the completion of phage liberation and then vanishes abruptly.

The time interval from the beginning of clearing to its completion is indicated. It falls well on the ascending part of the one step growth curve. In the one-to-one growth curves this ascending part is soon counter-balanced by the multiple adsorption loss, so that clearing *seems* to occur during a phase of little phage liberation.

were mixed, the phage would not infect all the bacteria, but distribute themselves according to the probability formulas derived by Poisson. If $P/B = n$ there will be a fraction e^{-n} of the bacteria uninfected. On the other hand our phage assays, though fully reliable as far as relative values go, are not as certain with respect to absolute value, because of the difficulty of obtaining an accurate determination of the efficiency of plating (*cf.* Ellis and Delbrück (1)).

It is not possible to make a complete quantitative prediction of the growth curve because it is not known in detail how the adsorption *en masse* of phage to a bacterium

that is already near a lysis from within will interfere with this process. It is also not quite certain whether those parts of the surface of the bacterium that adsorb the phage will lose their capacity of binding phage immediately upon lysis. In the strains used previously a slow decrease of phage assay after lysis could be ascribed to the continued "adsorption" of phage onto those scattered surface elements. No such decrease of phage assay was ever observed with the new strain. But such observations refer only to inactivation long after lysis and do not tell us whether the adsorbent is instantly destroyed upon lysis.

We have therefore calculated growth curves on the basis of two extreme assumptions.

(*a*) The amount of adsorbent decreases linearly from its initial value to zero during the 16 minutes in which the bacteria are lysed.

(*b*) The amount of adsorbent stays constant at its initial value throughout the course of lysis.

Case (*a*) is described by the differential equation

$$dP/dt = A - kB_0\,(1-t/T)\,P$$

In case (*b*) we have

$$dP/dt = A - kB_0P$$

In these equations the first term, A, represents the phage liberation by lysis during the interval T, as determined in the one step growth curves, the second term is the decrease of phage due to adsorption either on the unlysed bacteria only (case *a*) or on the unlysed bacteria plus the adsorbent from the lysed bacteria (case *b*).

These equations can be integrated explicitly.

We obtain in case (*a*)

$$P = \tfrac{1}{2}A\sqrt{\pi}\,\tau e^{2(T/\tau)^2-(T-t)^2/\tau^2}[G(T/\tau) - G([T-t]/\tau)]$$

with

$$\tau = \sqrt{2T/kB_0}$$

and $G(x)$ the Gaussian integral

$$G(x) = \frac{2}{\sqrt{\pi}}\int_0^x e^{-x^2}\,dx$$

In case (*b*), with constant adsorbent, the adsorption rate grows continuously with the free phage concentration. In this case we have therefore no point of inflection but a continuous asymptotic approach to the final titre

$$P = \frac{A}{kB_0}(1 - e^{-kB_0t}).$$

Since all required constants are known from independent experiments, the particular solutions applying to our case can be evaluated quantitatively. These have been plotted in Fig. 6. The experimental values fall between the limits set by these two cases.

We have also plotted the curve obtained in an ordinary one step growth curve, with B in excess (taken from Fig. 4). The difference between this

curve and the experimental values is the amount of phage lost by adsorption. The data show that about one hundred phage are lost per bacterium. It is clear that this loss depends entirely on the rate of adsorption, which is determined by the product kB_0. If we wish to increase the yield per bacterium we have to decrease either B_0 or k. Reduction of B_0 brings us to the condition employed in the one step growth curves. Here the maximum yield of phage growth per bacterium is obtained, but the actual concentrations of phage are of course very small.

A promising way of increasing the end titre of phage would be to reduce k, the adsorption rate constant. The very interesting experiments of Krueger and his coworkers (8, 9) on the influence of the addition of salts (NaCl and Na_2SO_4) to a growth mixture of phage and bacteria would seem to be completely in accord with the assumption that the adsorption rate constant is diminished in the presence of salt.

In fact, a diminished adsorption rate constant should manifest itself in several ways in a phage growth curve, in which one starts with low concentrations of both phage and bacteria. Namely

1. Delayed clearing, due to delayed adsorption, and therefore delayed phage growth.
2. Higher maximum concentration of bacteria, due to delayed lysis.
3. Higher end titre of phage, due to
(*a*) higher number of bacteria producing phage
(*b*) reduced loss of phage by multiple adsorption.
4. Higher ratio of free to bound phage during the stationary growth phase, due to the fact, that every phage particle spends a longer time in the free state between liberation and adsorption.
5. A period of constant bacterial concentration preceding lysis, when all bacteria are infected and cease to divide but when the phage concentration is not yet sufficient for lysis.

Precisely these five differences from the normal course and no others were noted by Krueger and Strietmann (9) in their study of the influence of the addition of Na_2SO_4.

SUMMARY

1. A new strain of *B. coli* and of phage active against it is described, and the relation between phage growth and lysis has been studied. It has been found that the phage can lyse these bacteria in two distinct ways, which have been designated lysis from within and lysis from without.

2. Lysis from within is caused by infection of a bacterium by a single phage particle and multiplication of this particle up to a threshold value.

The cell contents are then liberated into solution without deformation of the cell wall.

3. Lysis from without is caused by adsorption of phage above a threshold value. The cell contents are liberated by a distension and destruction of the cell wall. The adsorbed phage is not retrieved upon lysis. No new phage is formed.

4. The maximum yield of phage in a lysis from within is equal to the adsorption capacity.

5. Liberation of phage from a culture in which the bacteria have been singly infected proceeds at a constant rate, after the lapse of a minimum latent period, until all the infected bacteria are lysed.

6. If the bacteria are originally not highly in excess, this liberation is soon counterbalanced by multiple adsorption of the liberated phage to bacteria that are already infected. This leads to a reduction of the final yield.

The author wishes to express his appreciation for the hospitality extended to him by the Biology Department of the California Institute of Technology during the tenure of a Fellowship of The Rockefeller Foundation. In particular he wishes to record his indebtedness to Dr. E. L. Ellis for constant help and advice and to Mr. F. Gardner for technical assistance.

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STUDIES ON THE CHLOROPHYLLS AND PHOTOSYNTHESIS OF THERMAL ALGAE FROM YELLOWSTONE NATIONAL PARK, CALIFORNIA, AND NEVADA

By O. L. INMAN

(From the C. F. Kettering Foundation for the Study of Chlorophyll and Photosynthesis, Antioch College, Yellow Springs)

(Received for publication, March 6, 1940)

Much work has been done on the geological history of the hot springs and geysers, and several taxonomic studies of the flora and fauna of thermal water have also been made. The works of Weed (1), Setchell (2), Vouk (3), Brues (4), Copeland (5), and Howard (6), and references they cite, give a reasonably good survey of the findings in this field. The author has found but little physiological data on the chlorophyll-containing plants of thermal waters.

The main purpose of this study was to investigate the chlorophylls of algae growing in the hot springs and geyser pools of Yellowstone National Park and compare them with the chlorophylls found in other green plants growing at much lower temperatures. Studies of algae from hot springs in California and Nevada were also undertaken.

On March 6, 1937, through the courtesy of Mr. Joseph Joffe, Assistant Superintendent of Yellowstone National Park, the author received five samples of plants collected in the park on March 1 and shipped to Yellow Springs, Ohio, in glass bottles in the water in which they were found growing.

Taking a typical case the following data will indicate the nature of the study of each sample: Sample *Phormidium laminosum* (Ag.) Gom., collected from Mammoth area, temperature of water 65°C. Phase test and basicity test normal. Absorption spectra of algae in water:

I	II	III	IV	End abs.	Order of intensity: I, IV, II, III.
676	620	585	497	457	

Acetone extract after transference to ether gave the following absorption bands expressed in Ångstroms:[1]

I	Shadow	II	III	Shadow	IV	V	VI	End abs.	Order of
662.8	642.8	613.2	577.6	533.5	482.7	453.6	431.8	423.5	

intensity: IV, VI, I, V, III, II.

[1] None of the plant pigments were separated or purified due to the small quantities available.

The ether extract was saponified with 40 per cent methyl alcoholic potash. The absorption bands in the ether layer above the potassium chlorophyllin were:

I	II	End abs.	Order of intensity: I, II.
482.7	452.1	425.0	

Spectrum of saponified chlorophyll or potassium chlorophyllin in the methyl alcoholic KOH mixture:

I	II	III	End abs.	Order of intensity: III, I, II.
642.2	509.8	481.0	453.5	

Absorption bands of acidified ether extract or pheophytin:

I	II	III	IV	V	End abs.	Order of intensity: IV, I, V, III, II.
666.6	606.3	534.0	480.7	450.5	431.8	

Two other samples contained *Phormidium laminosum* (Ag.) Gom. and in one case *Spirulina labyrinthiformis* (Menegh.) Gom. mixed with *Phormidium*. All the above data apply to these samples. The temperature where these algae grew ranged from 48.5–65°C. One sample from the spring called Cleopatra (70°C.) consisted of sulfur bacteria only, although other samples contained Myxophyceae.

The most important features of these data were the abnormal ratio of chlorophyll *a* to *b* and the evolution of oxygen which took place when the plants were irradiated while at 20°C. This latter reaction would tend to indicate that such Myxophyceae normally growing at 65°C. could carry on the process of photosynthesis at normal temperatures. This would also show that light energy need not necessarily be supplemented by temperatures above 20°C. in order for these algae to carry on the process of photosynthesis for several days at least. No attempt was made to grow the algae at 20°C. or at higher temperatures.

After obtaining the above data, a collecting trip was made in Yellowstone National Park during July, 1937. Samples were taken from the Mammoth area, Madison Junction, Lower Geyser Basin, and Upper Geyser Basin. Care was exercised to collect from pools where the plants were continuously exposed to the hot waters for days at a time and not merely bathed intermittently with the hot water. Thirty-three samples were dried immediately (40–50°C.) and stored. In many cases some samples were dried and others preserved in formaldehyde. Some of these were examined on August 8 and 9, 1937, and repeated examinations made in October, 1938. Collections consisted largely of the calcareous or siliceous deposits to which the algae adhered.

In the truly siliceous areas of the park, nine samples were collected and examined. The absorption spectra, phase tests, and basicity tests of the extracted chlorophylls were normal. The ratio of chlorophyll *a* to *b* varied. Chlorophyll *b* was never absent but did not reach more than 15 per cent of the total chlorophyll present. The plants found were *Phormidium Treleasei* (Ag.) Gom. and *Synechococcus lividus* Copeland. The temperatures from which collections were made varied from 54.4–72.2°C. In one case sulfur bacteria were collected from waters 82.2°C. The elevation varied from about 7,000 to 7,500 ft.

A typical examination of these chlorophylls is given below: Sample No. 13A was collected near Green Pool, Upper Geyser Basin, July 15, 1937. The temperature of the water was 72.2°C. The collection consisted of *Phormidium Treleasei* (Ag.) Gom. and *Synechococcus lividus* Copeland. The phase test was normal with typical potassium chlorophyllin with an absorption band at 642 mμ. The basicity test was good. An acetone extract with transference of the chlorophyll to ether by means of water gave the following absorption bands:

I	II	III	IV	V	VI	VII	VIII	End abs.	Order of in-
663.4	644.5	613.0	578.0	535.5	483.0	456.0	433.0	410.0	

tensity: VIII, I, VI, VII, IV, III, V, II.

Pheophytin produced by adding 10 per cent HCl to the ether solution in the cold:

I	II	III	IV	V	VI	VII	End abs.	Order of intensity:
667.2	605.7	562.5	534.3	505.7	480.0	452.1	421.0	

I, VII, VI, IV, V, II, III.

Chlorophyll *b* was about 15 per cent of the total chlorophyll present. The examination of this material, which had remained in a dried condition in a glass jar, gave essentially the same chemical tests and the same absorption spectra when examined September 20, 1938.

From the calcareous Mammoth Hot Springs (elevation 6,500 ft.) area 20 samples were taken. The temperatures, where algae were found growing and collections made, ranged from 37–72°C. The pH of the waters varied from 6.5–6.7 in this region according to Copeland (5). The species included: (1) *Phormidium Treleasei* (Ag.) Gom.; (2) *Synechococcus lividus* Copeland; (3) *Spirulina labyrinthiformis* (Meneg.) Gom.; (4) *Phormidium laminosum* (Ag.) Gom.; (5) *Synechococcus elongatus* Nag. *Phormidium Treleasei* and *Synechococcus lividus* were often found together.

At Mound Terrace (Mammoth area) six collections were made from

waters with the following temperatures: 67°C.; 60°C.; 56.8°C.; 43°C.; 40°C.; 36.6°C. *Phormidium Treleasei* (Ag.) Gom. was a constant and the most abundant form present. In the three higher temperatures some *Synechococcus lividus* Copeland was found. It was quite clear that the temperature range of growth of these two species was not too restricted and examination of the chlorophylls gave normal chlorophyll and pheophytin absorption bands. Collections 11A, 7C, 4B, 4C, growing at temperatures from 59–67°C. and composed of the same genera and species as the samples immediately above, gave the following absorption bands when an acetone extract was transferred to ether: Sample 7C is representative of this group:

I	II	III	IV	V	VI	VII	VIII	IX	X	End abs.
664.0	644.0	610.9	576.5	548.8	532.5	501.0	481.2	455.5	432.5	420.0

Order of intensity: X, I, VIII, IX, VII, III, V, IV, VI, II.

The pheophytin absorption spectrum of 7C in ether was:

I	II	III	IV	V	VI	End abs.
667.0	606.8	548.5	534.5	509.5	472.0	453.5

Order of intensity: I, VI, V, III, II, IV.

In the samples 11A, 7C, 4B, and 4C there was an absorption band at about 548 mμ which is not present in any other known chlorophyll extract from plants. This also remained in the pheophytin spectrum. Whether this is some unusual accompanying pigment or a modified chlorophyll cannot be settled at the present time. It does not seem to be a matter of species or temperature at which the plants were growing, but it is worth observing that there was no such finding in the strictly siliceous sinter region of the Upper and Lower Geyser Basin. These modified chlorophylls appeared only in the calcareous waters. Such absorption spectra indicate another probable modification of the chlorophylls now known.

In the summer of 1939, studies of algae from "The Geysers" in Sonoma county, California, and at "Beowawe," Nevada, were made.

Material from The Geysers in California was collected from waters ranging in temperature from 49–66°C. All specimens showed the presence of a unicellular green alga which is probably *Chlorella sp.* The chlorophylls from these specimens were preserved in 5 per cent formaldehyde and upon examination gave normal pheophytin absorption spectra. This loss of Mg by the chlorophyll molecule during the storage of algae in 5 per cent formaldehyde is normal. It seems reasonable to assume that the chlorophylls of these green algae are also normal in respect to ordinary properties. To find no blue-green algae in the six specimens collected was rather unexpected.

The collections from Beowawe, Nevada, were taken from water of temperatures from 60–71°C. The elevation was about 4700 ft. The species represented were *Phormidium Treleasei* (Ag.) Gom. and *Phormidium laminosum* (Ag.) Gom. The chlorophylls were preserved in 5 per cent formaldehyde and spectroscopic examination showed a normal pheophytin spectrum in each case. Thus there appears to be no reason to assume that these chlorophylls were not essentially normal. It is interesting to note that the species here were also common in the Yellowstone material.

In all the habitats investigated, it was evident that the algae grew more abundantly in waters ranging in temperature from 37–50°C. This was also true of a single species such as *Phormidium Treleasei* (Ag.) Gom. In Yellowstone Park, where the altitude was from 6000 to 7500 ft., there would be a white to rose-colored thin mineral crust on many pools. Under this one often found considerable algae. This probably indicated that the radiation at the higher altitudes must have been too intense for these plants and they grew better where there was some shading. Nothing like this was found in the Geysers in California or at Beowawe, Nevada.

CONCLUSIONS

From the data obtained it seems most reasonable to conclude that the thermal Myxophyceae studied have, for the most part, normal chlorophylls with chlorophyll *b* constituting about half of the usual amount present in most green plants. Evolution of oxygen upon irradiation at normal room temperature was positive. These algae undoubtedly carry on photosynthesis in their natural habitat at the higher temperatures. What possible changes in the mechanism of the photosynthetic process might be found here must remain for future investigation to decide. Whether these species exist in thermal waters through adaptation or are relics of past ages cannot be definitely stated; but the fact that, for the most part, normal chlorophylls are present may add more credence to Vouk's contention that the flora of thermal waters is chiefly due to adaptation of these forms to high temperatures. After finding algae growing in intense radiation at high temperatures with the water often charged with hydrogen sulfide or containing arsenic, one feels that these plants are well worth further physiological study. An examination of the nature of the proteinaceous enzymes and the properties of many of the other chemical compounds associated with cell metabolism and photosynthetic reactions should be of much value.[2]

[2] All identifications of algae were made by Dr. Francis Drouet of the Field Museum, Chicago, Illinois.

SUMMARY

1. Myxophyceae normally growing at 65°C. evolved oxygen upon irradiation and showed evidence of retaining the power to carry on the process of photosynthesis at 20°C. This indicates that extra thermal energy is not essential for photosynthesis at least over a short period of time.

2. Chlorophyll *a* and *b* found in several species of Myxophyceae growing in waters ranging in temperature from 37–72°C. are essentially the same as found in plants growing all over the world. Certain standard chemical tests and spectroscopic examination of the chlorophylls were used as the criteria for these comparisons. The ratio of chlorophyll *a* to *b* often varied considerably but in general chlorophyll *a* showed an increase over the percentage found in most plants.

3. Green algae (*Chlorella sp.?*) were the only forms found at The Geysers, California. The temperature of the waters from which collections were made varied from 49–66°C.

4. Collections from Beowawe, Nevada were from waters ranging from 60–71°C. The algae belonged to the Myxophyceae and the species were like some of those found in Yellowstone National Park.

5. In some of the calcareous regions of Yellowstone National Park spectroscopic study of the chlorophylls revealed an unidentified absorption band at 548 mμ.

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REACTION TO VISUAL FLICKER IN THE NEWT TRITURUS

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I

The basis for recognition of visual flicker is described by the properties of the interrelationships of the flash frequency F and the flash intensity I which are critical for the exhibition of a forced reaction. The curve exhibiting the connection between F and I, as obtained from many repeated measurements, is a band defining a region on the $F - I$ grid within which there obtains a certain probability that succeeding equivalent determinations of critical F or I, under the same conditions, will be observed to fall.[1] The dependence of mean critical I upon F (or the converse but not identical dependence of F_m upon I) is fundamentally depicted by a probability integral in which F is ordinate and log I_m is abscissa.[2] For man and for various fishes the visual flicker curve is made up of two sections, a low intensity portion which rises to a maximum and then descends to $F = 0$, and a higher, larger part. The two parts overlap, additively, to varying degrees in different organisms. Many reasons have been held to support the view that the lower part represents the activity of those elements of excitation connected with the retinal rods, the upper with the cones.[3] The analysis of this situation is assisted by the consideration of further cases where, in different vertebrates, varying degrees of overlapping of the contributions due to rods and to cones may be expected and where these two groups of sensory elements are known to be present in varying relative proportions. The separation of the "rod" and "cone" curves on the log I axis may be great or small; the decay of the rod contribution as log I increases may be slow, delayed, or rapid; the maximum rod contribution may be comparatively large, or small. The ability of the analysis to account for the data in all these circumstances is a test of its capacity.

In a number of fishes examined differences of these types have been found

[1] Crozier, 1935–36; 1936; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*, *b*; 1937–38 *c*.

[2] Crozier, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *c*, *d*; 1937–38 *a*, *b*, *c*.

[3] Hecht and Verrijp, 1932–33; Hecht, Shlaer, and Smith, 1935; Hecht and Smith, 1935–36; Hecht, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1937; 1937–38 *a*.

(Crozier, Wolf, and Zerrahn-Wolf, 1937; 1937–38*a*), but they have been rather slight. The $F - \log I$ curve is of a form permitting easy separation of the two classes of elements. For man (Crozier, Wolf, and Zerrahn-Wolf, 1937–38*c*) the rod curve is relatively larger, and closer to the cone part; the degree of overlapping is more considerable, so that even at the lowest part of the data, down to $F = 1$, the separation is incomplete. A good part of the cone portion of the curve, however, is free from the addition of effects due to rods ($F = 18$ to 56, 59 in our measurements).

We now discuss a case in which the morphology of the $F - \log I$ curve is more extensively modified by the fusion of the segments due to the two classes of sensory elements. With the newt *Triturus* there is obtained a curve which cannot be interpreted in simple terms without careful analysis into its components. The lower magnitude of the integrated rod contribution, its small $\log I$ range, and its slighter separation on the I axis from that putatively due to the cones, produce in the curve of the summated end result a distortion which causes the $F - \log I$ graph to exhibit certain unusual features. The presence of more than one kind of rod and cone is clearly suggested by the histological structure of the retina of certain lower vertebrates. With *Triturus* the flicker curve shows a complexity which can perhaps be understood on this basis. Attempts to correlate the form of the composite flicker curve with details of retinal structure (and with ethological characteristics) must await wider examination of vertebrate types. In the frog[4] the complexity of the $F - \log I$ contour here described for *Triturus* is not found. Correlations of response data with known peculiarities of retinal structure do not of course imply in any necessary way that the quantitative properties of the data are determined at the retina.[5]

II

The water form of *Triturus viridescens viridescens* (Raf.) was used with the procedure previously described in connection with our observations on fishes, turtles, the gecko, and aquatic insects.[1] The newts were from a stock collected in western Massachusetts, but probably from different locations. They had been for some time kept at 4°C. in a cold room. After several days at room temperature, with good feeding, they were active and responsive. The 10 individuals selected were of about the same size, 3 to 3.5 inches in length. The experiments were made at 21.5°, with preliminary dark adaptation, by the routine already discussed.[1] The flash cycle was of equal light and dark intervals. During some days of preliminary trials there develops a definite conditioning to the experimental situation, such that the threshold response to flicker becomes clearer and more easily detected.

[4] Crozier and Wolf, 1939–40.

[5] *Cf.* Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1938; 1938–39.

The response is "slow," however, and by no means so convenient to work with as that shown by the fishes we have used. One usually obtains only a deflection of the newt's head in the direction opposite to that of the moving stripes. Rarely, walking or swimming with the stripes was seen. Its occurrence depends upon the general state of activity of the animal at the time, and not upon the intensity of light. Frequently tests must be repeated a number of times before reaction is obtained. The newts tend to become inactive in the thermostat, and gentle mechanical agitation was resorted to before the tests.

The adjustment of the intensity to the level critical for reaction, or of the flash frequency at fixed I, is made slowly. The response has a large latency, so that the possibility of overshooting the end-point is comparatively large.

Despite this difficulty, however, the variability in the measurements is not great. The procedure we have used as standard[1] takes three readings of I_c (or of F_c) in succession on one individual, averaged to give I_1 (or F_1). The mean dispersion of these individual I_c's is no greater than with other animals tested. The variation of I_1, however, is rather *less* than usually found; this is an artifact, due to the circumstance that one newt in the lot of 10 was persistently more sensitive and another less sensitive than the others. In more homogeneous groups of individuals the mean rank-order numbers for increasing magnitudes of I_1 are randomly distributed in successive tests.[1] With this lot of *Triturus* we were less fortunate in the effort to obtain homogeneity and equivalence of individuals. Thus in 36 sets of tests No. 8 gave a mean rank number $R_I = 2.82 \pm 0.240$ (P.E.$_M$), while No. 3 gave 6.97 ± 0.285. The difference is over 11 times its P.E., and is correlated with certain minor differences in the flicker curves as a whole. For the other 8 individuals used, the relative rank-order numbers have random values. For the exceptional animals the sensitivities are in the same order when F_c is measured. It is therefore a test of the value of the data that, despite the greater difficulty with which the readings can be made in comparison with the situation in some other animals used, small individual differences can be consistently identified over a long period of observation.

III

Table I contains a summary of the averages of the observations. The variation of I_1 follows the rule[1] that I_m and σ_{I_1} are in direct proportion (Fig. 1). Above $\log I = 0.5$ a different origin is required;[1] as in other flicker data on vertebrates, the point of transition to the high intensity (lower slope) section of the plot in Fig. 1 is rather definitely related to certain other properties of the measurements (section IV). The fluctuation of F_1 as measured by P.E.$_{F_1}$ also exhibits (Fig. 2) the qualitative properties expected from earlier work (Crozier, Wolf, and Zerrahn-Wolf, 1936–37*a*,*b*), in that it rises to a maximum at each region of inflection on the $F - \log I$ curve, while the final descending limb of the P.E.$_{F_1}$ curve falls to a level well above that from which it rises. The magnitudes of P.E.$_{I_1}$ and of P.E.$_{F_1}$ are of the order found with other forms.[1] It has been pointed out (section II) that one of the individuals in our lot of 10 showed a persistently higher

TABLE I

Mean critical illuminations for response to visual flicker at various fixed flash frequencies F, with $t_L/t_D = 1$, as log I_m and log P.E.$_{.1I_1}$ (millilamberts), for the newt *Triturus viridescens viridescens* (Raf.); and mean F_1 with P.E.$_{.1F_1}$ (flashes *per* second) at fixed intensities. Temperature, 21.5°. $N = 10$ individuals, three observations on each point ($\Sigma n = 30$). In view of the peculiar shape of the flicker contour, a second set of 10 *Triturus*, of the same size, was studied, some 6 months later; the entries under log I_m and log P.E.$_{.1I_1}$ in parentheses were obtained with this second series; they agree quantitatively with the data from the first set.

F	log I_m	log P.E.$_{.1I_1}$	log I	F_m	P.E.$_{.1F_1}$
per sec.	*millilamberts*	*millilamberts*	*millilamberts*	*per sec.*	*per sec.*
2	$\bar{6}.9938$	$\bar{7}.7166$			
			$\bar{5}.000$	**1.96**	0.0321
3	$\bar{5}.3332$	$\bar{6}.0282$			
			$\bar{5}.500$	**3.77**	0.0330
4	$\bar{5}.5553$	$\bar{6}.2090$			
5	$\bar{5}.7482$	$\bar{6}.7398$			
			$\bar{4}.000$	**5.85**	0.0103
6	$\bar{4}.0257$	$\bar{6}.4926$			
7	$\bar{4}.4153$	$\bar{5}.0358$			
			$\bar{4}.500$	**7.36**	0.0446
			$\bar{3}.000$	**7.94**	0.0526
8	$\bar{3}.0806$	$\bar{5}.9258$			
			$\bar{3}.500$	**8.68**	0.0312
9	$\bar{3}.5974$	$\bar{4}.3143$			
10	$\bar{3}.8238$	$\bar{4}.7171$			
	($\bar{3}.8240$	$\bar{4}.0457$)			
			$\bar{2}.000$	**11.72**	0.0615
12.5	$\bar{2}.0906$	$\bar{4}.4839$			
15	$\bar{2}.3608$	$\bar{3}.3078$			
	($\bar{2}.3623$	$\bar{4}.8684$)			
			$\bar{2}.500$	**16.44**	0.358
17.5	$\bar{2}.6296$	$\bar{3}.1274$			
20	$\bar{2}.9319$	$\bar{2}.0175$			
	($\bar{2}.9287$	$\bar{3}.2646$)			
			$\bar{1}.000$	**20.12**	0.372
22.5	$\bar{1}.2368$	$\bar{3}.6814$			
23	$\bar{1}.3197$	$\bar{3}.8108$			
			$\bar{1}.500$	**24.02**	0.318
25	$\bar{1}.5677$	$\bar{2}.3738$			
	($\bar{1}.5642$	$\bar{3}.8874$)			
26	$\bar{1}.7250$	$\bar{2}.2707$			
27.5	$\bar{1}.9353$	$\bar{2}.4473$			
			0.000	**27.08**	0.375
29	0.1738	$\bar{2}.6548$			
30	0.4012	$\bar{1}.3043$			
	(0.3634	$\bar{2}.8973$)			
			0.500	**30.80**	0.214

TABLE I—*Concluded*

F	$\log I_m$	$\log \text{P.E.}_{I_1}$	$\log I$	F_m	P.E._{F_1}
per sec.	*millilamberts*	*millilamberts*	*millilamberts*	*per sec.*	*per sec.*
31	0.5625	$\bar{1}.1342$			
32.5	0.7925	$\bar{1}.0409$			
33	(0.8590	$\bar{1}.1878$)			
			1.000	33.53	0.254
35	1.2603	$\bar{1}.8988$			
	(1.2180	$\bar{1}.3892$)			
36	1.4108	$\bar{1}.7714$			
			1.500	36.45	0 214
37.5	1.7621	0.2039			
			2.000	38.93	0 251
39	(2.1028	0.2679)			
40	2.3170	0.7177			
	2.3124	0.1568			

(No. 8) and another (No. 3) a persistently lower relative sensitivity. The figures in Table I are based on the measurements with all 10. Indices of scatter (P.E._{I_1}) computed with these two omitted, the eight remaining ones having randomly distributed mean rank-order numbers for relative sensitivity, show but an insignificant change from those given in Fig. 1. It is of some interest that, despite the slowness of the response of *Triturus*, the method of observation is sufficiently delicate to reveal slight consistent differences between individuals when they occur. This is consistent with the findings with *Anax* (1937–38*b,d*) and other forms.[1] The mean variation in successive readings with one individual is of the order of 0.4 per cent. There is no systematic change in the relative sensitivities with time during the long period of observation (about 1 month); the observations were arranged to reveal any such changes.

The F_m figures do not lie definitely above the $F - I_m$ curve (Fig. 3), as found with *Anax* and sunfish (1936–37*a,b*). The slowness of the newt's response might lead to this result, since the danger of "overshooting" to too low an F_c might be expected,—particularly if the level of adaptation is higher than when I_c is being determined. The three successive readings on each individual show no temporal drift, so it is unlikely that adaptation is a factor. In the region $\log I = \bar{2}.3$ to 0.5 the chief discrepancies between F_m *vs.* $\log I$ and $\log I_m$ *vs.* F occur. It is to be noted that in this zone the curve has a special character (section IV). The points in Fig. 3 are averages. Consideration of the individual measurements discloses that it is characteristically in the range from $\log I = \bar{2}.0$ to 0.5 that the F_m readings are too low, rather than elsewhere. We may therefore suspect that some

special feature of the case in this region is responsible. There is other evidence for this.

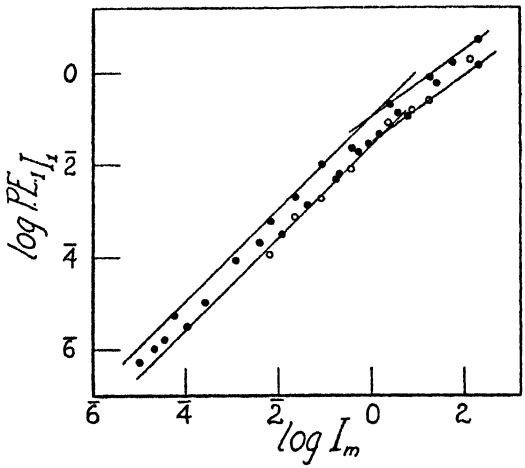

FIG. 1. I_m and P.E.$_{I_1}$ are directly proportional, up to log I = 0.5, as the log plot is rectilinear with slope = 1. I_m is the mean critical intensity for response of *Triturus* to flicker, at various flash frequencies; P.E.$_{I_1}$ is the P.E. of the dispersion of I_1. Above 0.5, the slope changes; as in other flicker data on vertebrates the terminal part of the graph can be rectified with slope = 1 by taking a new origin.[1] (The open circlets are points obtained in the second series of measurements; *cf.* Table I.)

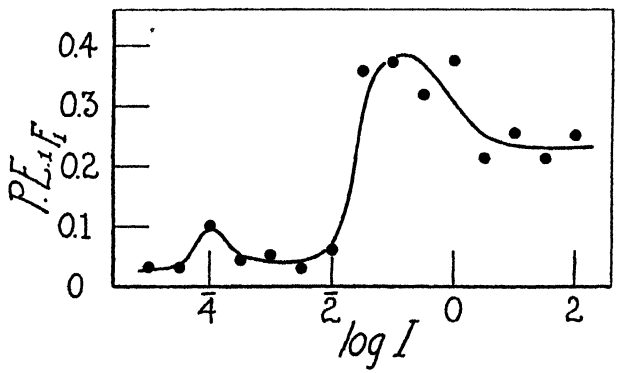

FIG. 2. P.E.$_{F_1}$ *vs.* log I

IV

The plot in Fig. 3, as well as the graphs for the data of the several individuals separately, show separation into two main portions: a low intensity part and a steeper, larger part at higher intensities. Through the second

of these no simple smooth curve can be put. The change in curvature at about log I = 0.5 is real and significant. A probability integral is easily

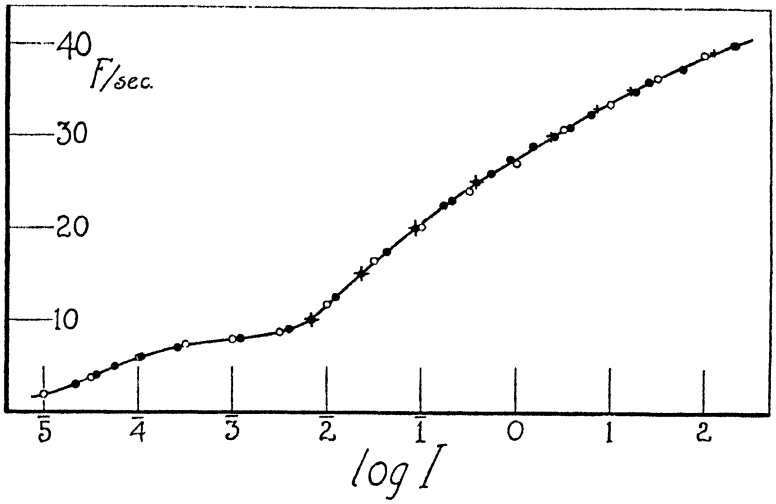

FIG. 3. F *vs.* log I for response of *Triturus* to flicker. Data in Table I. Solid dots, I_m; open circlets, F_m. Each point is an average of 30 determinations. Crosses, I_m from a second, independent series of measurements (Table I).

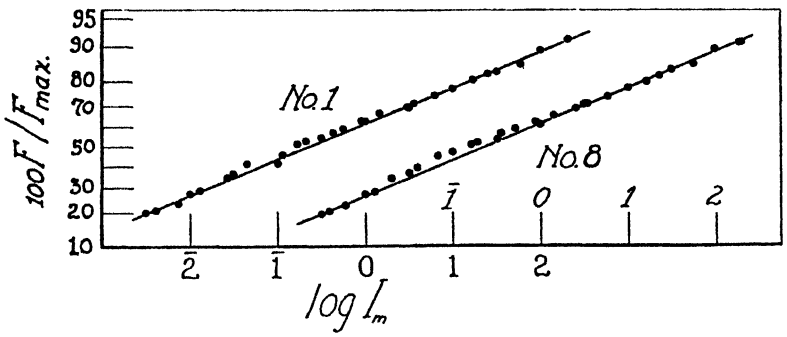

FIG. 4. F *vs.* log I for the upper (cone) portions of the data on two individuals, Nos. 1 and 8, which show about the widest difference in the extent of the departure from a probability integral in the neighborhood of log $I = \bar{1}$. Each point is the mean of three measurements. The slope is the same for No. 1 ($F_{max.}$ = 43.7) and for No. 8 ($F_{max.}$ = 44.0). Discussed in the text.

adjusted to the points above this intensity, but between log $I = \bar{2}.0$ and 0.5 there is a definite departure from it. The departure cannot be traced to properties of the apparatus or procedure. No filters were used to regulate intensity above log $I = \bar{2}.0$. It is important that the departure differs in extent for the several individuals (Fig. 4), and that the uncharacter-

istically low F_m readings in this zone tend to approach the probability integral (Figs. 4 and 5). The departure from the probability integral is thus of a nature quite different from that which we have analyzed in the responses of *Anax* (1937–38*b*).

If one separates the rod function curve from the composite total (Fig. 5), on the basis that the uppermost part of the data is properly described by a probability integral, the result is of the same type as that which we have

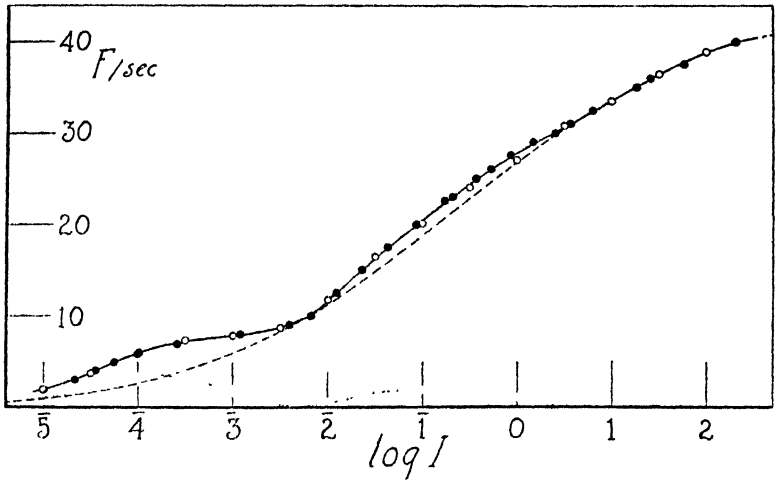

FIG. 5. The flicker response contour for *Triturus*, analyzed with reference to the criteria discussed in the text. Open circlets, F_m; solid, I_m. The dashed line is a probability integral. The left-hand dotted line (by difference) gives the course of the rod contribution to F as I increases. The bulge between log $I = \bar{2}.0$ and 0.5 is taken to be due to the activity of a different subsidiary group of receptor units (dotted curve at the right).

already described for the measurements with various fishes (1936–37*d*; 1937–38*a*) and man (1937-38*c*). The raw figures for the rod-dominated lower segment of the observations do not at all adhere to a probability integral. The ascending and the descending parts of the rod curve obtained by difference (left-hand dotted curve in Fig. 5) are quite precisely described in this way, as in the various cases already analyzed.

The rod component of the flicker reaction contour for *Triturus* does not differ materially from that obtained with certain fishes at the same temperature (1937–38*a*), save that it has a lower $F_{max.}$ and a quicker decline. Its location as measured by log I at the inflection point ($\bar{5}.4$) is slightly higher than that for the fishes tested, its S.D. ($\sigma'_{\log I}$) about the same, its declining curve a little steeper. The rod curve inflection is separated from that of the cone member by about the usual distance on the log I scale.

The one unusual feature of the cone curve, aside from the complicating bulge between log $I = \bar{2}.0+$ and 0.5, is its great spread. Its S.D. is about twice that found for the several kinds of teleosts we have tested, and is also larger than that obtained with man (1937–38*c*). The maximum assigned, and the value of log I at the inflection, are very similar to those for the fishes.

The justification for the use of the curve in Fig. 5 is (1) that it describes the upper part of the data, (2) that it provides a rational interpretation of the rod region of the data, and (3) that the degree of departure from the probability integral between log $I = \bar{2}.0+$ and 0.5 is a function of the individual newt. The nature of this departure (Fig. 5) is such as to suggest the presence of a third kind of visual element in the retina—possibly "double rods"—present in about one-half the frequency of the typical rods. The additional elements are probably[6] not due to photoreceptors in the skin, as experiments with newts having the eyes covered by special "blinders" gave no reactions at any intensity for flash frequency. The behavior of the F_m figures in this region of the curve could be interpreted as indicating that these elements have special properties as concerns photic adaptation. The presence of special kinds of visual cells in developing amphibians is of course not unknown. There is a possibility that the number and properties of these "extra" elements should differ more extensively from one individual to another than do those of the more typical receptor units. It is to be noted that, as with other vertebrates (1937–38*d*), the break in the log I − log P.E._1 curve (Fig. 1) comes at the level of intensity (here, log $I = 0.5$) where the contribution of "rod" elements fades out completely.

The peculiar bump on the flicker response curve (Fig. 3) was confirmed by a separate series of experiments, 6 months later, with a new lot of animals. These data are also given in Table I, and in Figs. 1 and 3. In this series, as for the earlier one, several individuals are found to be persistently more excitable than the others.

We are indebted to Dr. Gertrud Zerrahn-Wolf for assistance in the experiments.

SUMMARY

The flicker response curve for the newt *Triturus viridescens* (water phase) has much the same quantitative structure as that found with various fresh-water teleosts at the same temperature (21.5°). The variability of critical

[6] It is of course not inconceivable that over a certain range of flash intensities visual effects originating from the eyes could be reinforced by impulses originating in the skin; such effects could not be detected in the absence of retinal stimulation.

intensity and of critical flash frequency likewise follows the same rules. The cone portion of the $F - \log I$ curve is much more widely spread, however. This, and the rather low maximum to which the rod curve rises, produce a considerable overlapping of the two parts additively fused.

In addition, and to an extent which differs in various individuals, there is apparent a slight departure from the probability integral form of the cone curve. Reasons are given for considering that this is possibly connected with the rôle of an additional (small) number of (perhaps temporary, or developmental) retinal elements in addition to the typical rods and cones.

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THE FLICKER RESPONSE CURVE FOR FUNDULUS

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I

A number of vertebrates have been examined by us as to the form of the interdependence of flash frequency (F) and flash intensity (I) for reaction to visual flicker. The technic, apparatus, and procedure have been uniform in all these cases. There has been used, except in special experiments, a flash cycle in which light time and dark time are equal, and the temperature is the same (21.5°). We know, for several forms, that the *shape* of the F-log I curve (the flicker response contour) is not a function of temperature,[1] nor of the fraction of the flash cycle occupied by light.[2] Its position on the log I axis is governed by the temperature; its position and the magnitude of the F-scale units are dependent upon the light time fraction of the cycle time. Its shape is, however, a function of the nature of the animal tested.[3]

For typical vertebrates the flicker response contour comprises two parts or segments, so that the curve is a doubly inflected S.[4] The association of the smaller, low intensity part with the functional activity of retinal rods and of the larger, upper portion with that of retinal cones[5] is consistent with a large body of speculation and systematized information in visual physiology.[6]

In keeping with this conception, an animal which exhibits no histologically recognizable retinal rods, the turtle *Pseudemys*, and another with no cones, the gecko *Sphaerodactylus*, give flicker response curves which follow a

[1] Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*, *b*; 1937–38 *b*; 1938–39; Crozier and Wolf, 1940; 1939–40 *a*.

[2] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*, *e*; Crozier and Wolf, 1939–40 *b*.

[3] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*; 1937–38 *a*; 1937; 1938–39; Crozier, 1937; Crozier and Wolf, 1938–39 *a*; 1939–40 *a*.

[4] Wolf and Zerrahn-Wolf, 1935–36; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; 1937–38 *a*, *b*, *e*.

[5] Hecht, 1937 (review); Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; 1937–38 *b*, *e*; Crozier and Wolf, 1938–39 *a*, *b*, *c*.

[6] Hecht, 1937.

simple, uncomplicated sigmoid course over its whole extent.[7] To this curve there can be fitted a probability integral

$$F = kF_m \int_{-\infty}^{\log I} e^{-(\log I/I_i)^2/2\sigma^2_{\log I}} \cdot d \log I_m$$

which describes the interdependence of F and I with excellent fidelity.[7]

This equation also serves to describe each segment of the compound curve for other vertebrates (various teleosts,[8] and man[9]). The "rod" and "cone" curves overlap in a particular way,[8, 9] which has been analyzed by the assumption that after reaching a maximum value of F the rod curve declines with increasing intensity. Various bits of evidence, in addition to its descriptive utility, support this idea.[5] It is of some moment, however, that this general formulation be tested by applying it to a variety of animals. We have pointed out[3] that different kinds of overlapping of the rod and cone contributions to the F-log I contour are to be expected, and some of these have since been found.[8, 9] The possibility cannot be lost sight of that there might well be discovered in certain vertebrates evidence of a third group of effects, in addition to those labelled as due to rods and to cones respectively; such as might for example be traceable to a distinct rôle of parafoveal cones, or of double or twin rods or cones in lower forms. A distinct suggestion of this is given by our measurements with the newt *Triturus*.[10]

Our immediate interest, however, is not with the bearing of such information upon the duplexity doctrine,[11] nor with ethological parallelisms between habits and visual performance in response to flicker. The elements of excitation involved in threshold or marginal response to flicker give evidence, in most vertebrates, that they form two groups or populations. This does not mean that the details of the form of the flicker contour are due to or that they measure properties of the peripheral retina. The response is a reaction of the whole organism, and there is every reason to believe that the elements of excitation, whose effects are the basis of the measurements, are central nervous elements. The initiation of their activities is by way of their peripheral (retinal) representatives, and if these are of two or more categories there could well be corresponding groups of

[7] Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1938–39 *b*.

[8] Crozier, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*; Crozier and Wolf, 1938–39 *a*.

[9] Crozier, 1937; Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *b*.

[10] Crozier and Wolf, 1939–40 *b*, *c*.

[11] Crozier, Wolf, and Zerrahn-Wolf, 1938–39; Crozier and Wolf, 1938.

the central elements. Where rods and cones are distinguishable, the correlation with classes of elements concerned in the determination of the index-response to flicker is likely to be useful. But it is equally possible that instances should occur in which a composite flicker contour is obtained with an animal having no detected histological duplexity of retinal constitution.

The chief utility of further data upon the F-log I curves of diverse animals is to obtain a test of the generality of the method of analysis of the composite contours. Breeding experiments show pretty clearly[12] that the rod and cone populations of effects, as we may for convenience continue to label them, are independently determined. Since the shape of the F-log I curve is demonstrated to be a constitutional property of the organism[13] we can inquire as to the correlation of the properties of rod and cone effects in the different curves. This can be done quantitatively in terms of the three parameters of the probability integral describing each partial curve—the values of $F_{\text{max.}}$, log $I_{\text{infl.}}$, and $\sigma_{\log I}$—and of the degree of separation between the rod and cone curves.

Data on the teleost *Fundulus heteroclitus* were obtained with these purposes in mind, and also with the idea that it should be a form suitable for experiments seeking to modify the quantitative properties of the F-log I curve. While *Fundulus* has proved to be in some respects a difficult animal to work with, the measurements do give information upon some of the points in view.

II

The general procedure followed exactly the outlines of that which we have followed with other organisms we have tested. An important feature of this technic is the maintenance of the healthy condition of the individuals tested, and their continued activity after habituation to laboratory conditions and to the manipulations involved in the tests. From the standpoint of the observer *Fundulus* has been a rather more "difficult" animal to work with than certain other teleosts we have used.

About 50 *F. heteroclitus*, obtained from a dealer in Boston in September, 1937, were kept in three tanks with Southern exposure, for 3½ months. They were maintained in fresh water, and tested in this medium. When bought they ranged in length from 1.75 to 3.0 inches. They grew considerably in the laboratory; about twelve died.

For observation, each *Fundulus* was first put into a separate jar. They were for some time hyperreactive to nearby movements. Each individual was tested in the apparatus, at a flash frequency of $F = 15$/sec. For the subsequent tests the ten most consistently reactive individuals were selected. At first the fishes were "restless," reacted to chance

[12] Crozier, Wolf, and Zerrahn-Wolf, 1937; 1937–38 *a*; Crozier and Wolf, 1938–39 *a*.

[13] Wolf and Zerrahn-Wolf, 1935–36; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*; 1937–38 *a*; Crozier and Wolf, 1938–39 *a*.

vibrations, and scarcely came to rest after once being exposed to visual flicker. After continuance of the routine procedure for about a week—feeding at a fixed time, transferring to observation jars, placing in the thermostat for 2 hours' dark adaptation, and transference to the apparatus for exposure to flicker—the behavior of these individuals became more stabilized. The reactive state was found to be distinctly improved by keeping the individual aquaria in a room in which people were frequently moving about.

After being "broken in" in this way these fishes come to the surface of the water when the covers of their aquaria are lifted and jump at offered food. From this time onward they give, during tests of response to flicker, reactions which are as precise as those with any of the number of teleost species we have examined.

The test for critical flash illumination consists in exposing an individual in its aquarium, surrounded by a rotating striped cylinder, to a gradually increased illumination controlled by a manually operated diaphragm.[13] The speed of rotation of the striped cylinder determines the flash frequency of illumination of any fixed point. This speed is kept constant by observation of a sensitive voltmeter measuring the current produced by a suitable magneto geared to the shaft of the motor driving the cylinder. The motor speed can be controlled to a precision of 0.1 F/sec. under the most unfavorable conditions of stripe number on the cylinder.

From calibration curves giving the intensity of illumination at the position of the fish as a function of diaphragm opening, the illumination which is achieved at the moment of the fish's reaction to the movement of the encircling stripe system is read.

The response of the animal consists in a suddenly aroused swimming motion in the direction of the movement of the stripes. This response appears sharply as soon as the intensity of illumination (with F fixed) has been increased to the critical level. Backward swimming, observed more frequently with sunfishes, almost never occurs with *Fundulus*. Rarely, a *Fundulus* directly facing the wall of its container will "back off" a little and then follow the motion of the stripes. Should an individual already be in motion when the light diaphragm is opened, the direction of its movement is clearly altered when responsé to seen flashes occurs. In most such cases tests were repeated, and readings taken only when the fishes were quiet. The jerky response at threshold I is then unmistakable. Observations at low illuminations were preceded by about 1 hour of dark adaptation of the observer. In general, the ease of judgment of the occurrence of a threshold response to flicker was less above $F = 25$ than below. This condition is different from that obtaining with certain other forms. It does not affect the quantitative behavior of the scatter of the readings of mean critical intensities for the different individuals. This scatter, here measured by P.E.$_{I_1}$, obeys precisely the same law as that which we have found to hold for other organisms.

Three readings of critical illumination were obtained for each individual, in succession, at each fixed flash frequency. Ten individuals were used throughout. The mean of each set of three readings was taken as I_1, the mean of the ten averages being recorded as I_m. This is the procedure we have consistently employed in flicker response observations with lower organisms. The reasons for it are discussed in our earlier papers. The order of the levels of F selected was so arranged as to reveal any time-drift in sensitivity of the animals used, or in the observer's appreciation of the movements constituting the index response of the fish. The statistical examination of the records reveals no evidence that such effects are present in the data.

The measurements were made with *Fundulus* kept in fresh water. The temperature

was 21.5° during the tests. The flicker cycle used involved equality of light time and dark time ($t_L/t_D = 1$). Supplementary tests at several appropriate flash frequencies were made with *Fundulus* living and exposed in sea water. (These results are not entered in Table I or in Fig. 1.) No differences in the properties of I_m were detected for the two series.

TABLE I

Mean critical illuminations for reaction of *Fundulus heteroclitus* to flicker as a function of flash frequency, F/sec., computed as described in the text, with the measures of the dispersions of I_1. Temperature = 21.5° C. Equality of light time and dark time in the flash cycle ($t_L/t_D = 1.0$).

F/sec.	log I_m	log P.E.$_{I_1}$
1	$\bar{7}.9777$	$\bar{8}.6371$
2	$\bar{6}.3808$	$\bar{8}.9267$
3	$\bar{6}.6833$	$\bar{7}.3516$
4	$\bar{6}.9353$	$\bar{7}.2418$
5	$\bar{5}.1623$	$\bar{7}.4788$
6	$\bar{5}.4012$	$\bar{6}.0792$
7	$\bar{5}.6688$	$\bar{6}.2250$
8	$\bar{4}.0913$	$\bar{6}.5943$
9	$\bar{4}.6310$ $\bar{4}.5611$	$\bar{5}.2732$ $\bar{6}.9940$
10	$\bar{4}.9268$	$\bar{5}.3890$
11	$\bar{3}.3477$	$\bar{5}.9267$
12	$\bar{3}.6807$	$\bar{4}.4501$
15	$\bar{2}.1755$	$\bar{4}.5001$
17.5	$\bar{2}.5161$	$\bar{3}.0390$
20	$\bar{2}.8214$	$\bar{3}.3073$
25	$\bar{1}.3664$	$\bar{2}.0881$
30	$\bar{1}.8543$	$\bar{2}.4997$
35	0.2610	$\bar{1}.0633$
40	0.6719	$\bar{2}.9972$
45	1.1361 1.1281	$\bar{1}.3797$ $\bar{1}.3747$
48	1.4746	$\bar{1}.6910$
50	1.8226	0.2151
52	2.2669	0.1230

III

The data secured by the procedure outlined in section II are summarized in Table I.

When log I_m is examined as a function of F it is seen to have the general form of the curve obtained with other fishes we have tested. The curve (Fig. 1) is a double S-shaped affair, but the slope of its greater part does not superficially have exactly the properties previously encountered. The analysis of this situation confirms in a rather striking way the validity of

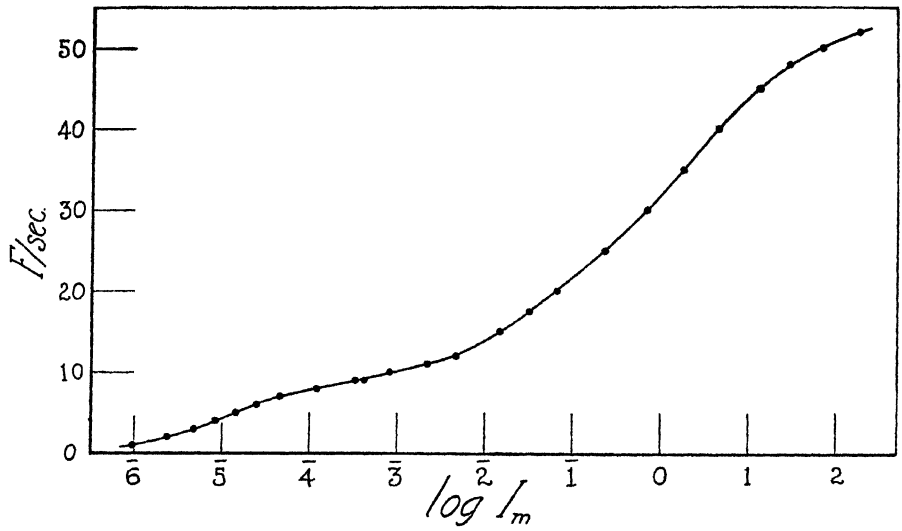

FIG. 1. The flicker response contour for *Fundulus heteroclitus*, log I_m *vs.* F: at 21.5°, in fresh water, with equality of light time and dark time in the flash cycle. Data in Table I.

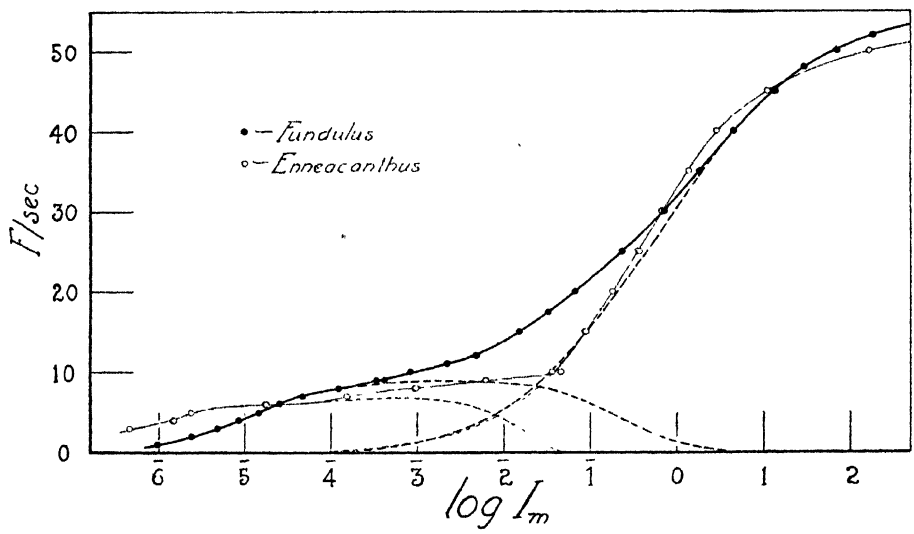

FIG. 2. Analysis of the flicker response curves for *Fundulus* and for the sunfish *Enneacanthus*,[3] under the same conditions. In each case the composite curve is resolvable into two parts: the rod portion at lower intensities, falling off as higher intensities are required, and the cone part. These parts separately adhere to probability integrals (Figs. 3 and 4). The rod part is large with *Fundulus*, and distorts the curve in a comparatively extreme way. The cone curve for *Fundulus* most nearly approaches that for *Enneacanthus* among the forms studied. The independence of rod and cone contributions is made evident by the fact that their properties vary quite independently in the different animals whose flicker response curves have been determined.

the analytical method we have used for the description of the various flicker response curves which have been measured. Among the known curves of this kind the graph for *Fundulus* provides a case which is important because of its rather extreme character.

The flicker response contours of a variety of animals have been described with high fidelity by means of probability integrals

$$F = kF_{\max.} \int_{-\infty}^{\log I} e^{-(\log I/I_0)^2/2\sigma^2_{\log I}} \cdot d \log I,$$

where I_o is the intensity at the inflection point of the curve. For the turtle *Pseudemys*,[7] which has retinal elements of one type only (cones), such a curve adequately fits the data from end to end of the intensity scale. With arthropods such as *Apis*[14] and *Anax* (larva)[14] the gross morphology of the optic surface introduces complications which have been resolved by other experiments.[14, 15] The reasons for the applicability of a probability integral are connected with the interpretation of response to visual flicker as a limiting case of the discrimination of intensities.[16] The inclusive theory of intensive discrimination,[17] which embraces sensorial phenomena of tension,[18] superficial pressure,[19] and auditory[20] response, and presumably other kinds of sensory phenomena, appears to require that the discrimination of intensive effects involves essentially the statistical comparison of populations of elementary resultants for which the compared intensities are respectively responsible.

The flicker response curves for one organism, man, have been fitted by means of equations derived from the theory that the quantitative properties of such measurements are governed by the photochemistry of the retina.[6] The resulting equations do not describe the data as well as probability integrals do, and the properties of their parameters as revealed in appropriate additional experiments are inconsistent with the facts.[1, 2] The responses which are the basis of the measurements involved are reactions of the organism as a whole. The assumption that they are governed by the state of the peripheral retina is unnecessary, and in certain crucial cases[2] can be shown to be inadmissible. The equation derived on the basis of an immediate reflection of retinal events appears to give a reasonable description of the data for the human observer because it is formally identical with a logistic in log I;[21] the latter cannot usually be separated by

[14] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*.
[15] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *e*.
[16] Crozier, 1935; 1935–36.
[17] Crozier, 1936; Crozier and Holway, 1938.
[18] Crozier and Holway, 1937; Holway and Crozier, 1937 *a*.
[19] Holway and Crozier, 1937 *a*, *b*.
[20] Crozier and Holway, 1937; Upton and Crozier, 1936; Holway and Hurvich, 1937.
[21] Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *c*.

curve fitting from a probability integral (in log I) except within 2 per cent or so distance of the asymptotes. For other organisms the assumptions underlying the derivation of the photostationary state equation, as applied to its adjustment to the observations, become unreasonable.[8] The retinal theory of the control of the properties of the flicker curve in addition fails to give a satisfactory account of the composition of the duplex curve for most vertebrates,[5, 7] whereas the analysis by probability integrals does.[7, 8]

Among lower forms thus far examined, the flicker response curve of *Fundulus* is comparatively extreme. At one end of the series we have the turtle *Pseudemys*,[7] for which the curve is a simple probability integral without complication. This is correlated with the absence of retinal rods. The presence of double or twin cones does not involve or lead to the activity of more than one detectable population of sensory effects. Several teleosts,[12] possessing retinal rods, exhibit increasing degrees of prominence of a subsidiary population of effects at lower intensities. The rising curve of these (rod) effects is also well fitted by a probability integral. By subtraction of the ordinates of the larger curve attributed to the exclusive rôle of retinal cones a curve of "decay of effect due to rods" is obtained; it is a reversed probability integral.[8] The contribution due to the participation of rod-connected elements concerned with response to flicker declines systematically, and in terms of a population of excitable elements, as the critical intensity for threshold total effect increases.

For the newt *Triturus*[10] and for *man*[9] the rod contribution curve (of different magnitude in the two cases) so to speak, sits upon the lower end of the cone curve.

In all of these cases the resolution of the duplex curve is obtained quantitatively on the basis that the fundamental function for each of the two groups or populations of effective elements is described as a probability summation.

The curve for *Fundulus* (Fig. 1) requires a comparatively considerable enlargement of the rod-controlled contribution to the total observable flicker sensitivity. The rod contribution also continues to be evident at higher intensities than with fishes earlier tested. This results in a very marked distortion of the curve in the region where presumptive cone effects are dominant. The uppermost part of the data is, however, very accurately fitted by a probability integral (Figs. 2 and 3). So also is the lowermost rising portion. For other fishes thus far tested the entrance of the effects due to cones produces a distinct "bump" on the composite graph.[8] With *Fundulus* the junction is on the flat region of maximum F for the rods, and is not perceptible as a distinct irregularity.

The difference between the curves for *Fundulus* and for other teleosts is strikingly shown in Fig. 2. Comparison is there made with the observa-

tions on the sunfish *Enneacanthus*,[22] at the same temperature. The cone curve for *Enneacanthus* most closely approaches that for *Fundulus*, among the others available. The contrast in the character of the rod contributions

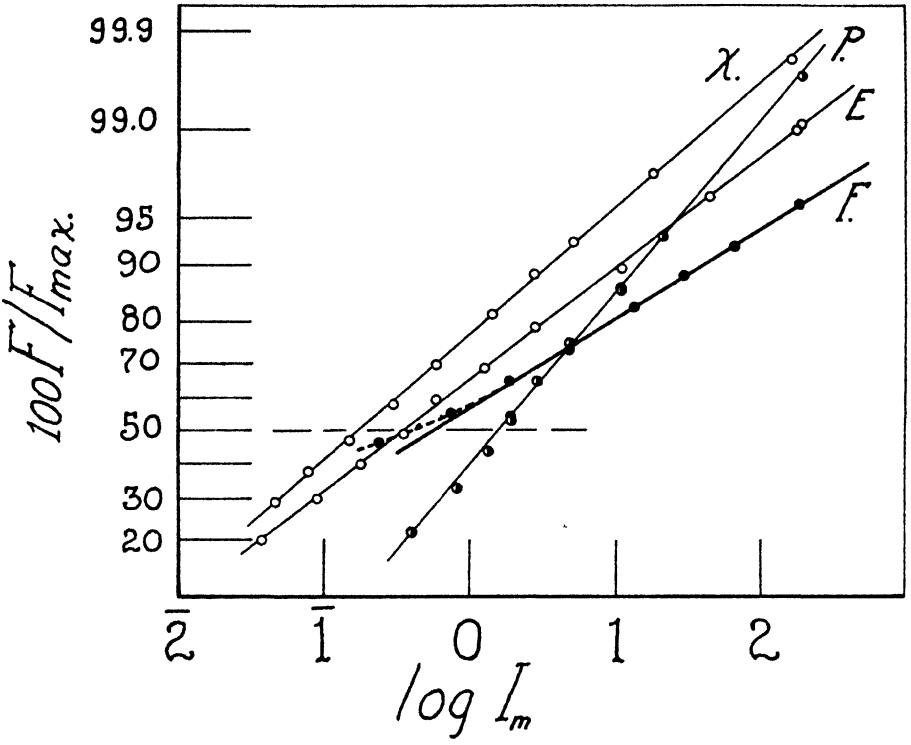

FIG. 3. The cone portions of the flicker response curves for several teleosts[3] are compared upon a probability grid. For *Fundulus* (*F.*) only the uppermost 40 per cent of the *F.* range is described by a probability integral; for the others the degree of overlapping with the rod contribution is less extensive, so that with *Enneacanthus* (*E.*) some 80 per cent of the *F.* range is uncomplicated. The lines drawn have been extrapolated to give (Fig. 2) a means of dissecting the composite graph into rod and cone contributions. (In all cases, temperature = 21.5° and $t_L/t_D = 1$.) *F.*, *Fundulus*; *E.*, *Enneacanthus*; *X.*, *Xiphophorus*; *P.*, *Platypoecilius*. The values of $F_{max.}$ are: *F.*, 54.2; *E.*, 52.1; *X.*, 43.1; *P.*, 46.1.

is pronounced. Each of the duplex curves has been analyzed (Fig. 2) by the procedure used for other forms. The result is given in Fig. 2. The ascending and descending rod curves and the cone curve are calculated probability integrals. The degree of adherence of the data to the corresponding equations is adequately shown by the graphs in Figs. 3 and 4.

[22] Wolf and Zerrahn-Wolf, 1935–36; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*, *c*, *d*; 1937–38 *d*.

The parameters of the probability integral are rationally related to (1) the genetic composition of the animal,[8] (2) the temperature,[1] and (3) the proportion of light time in the flash cycle[23] $(t_L/(t_L + t_D))$. They are therefore proper indices of the properties of the flicker contour. For log I_m *vs.* F these parameters differ in a characteristic way from one animal to another (Figs. 3 and 4). It is natural to inquire if correlations can be established between parameters of the rod and cone functions. This might well be a

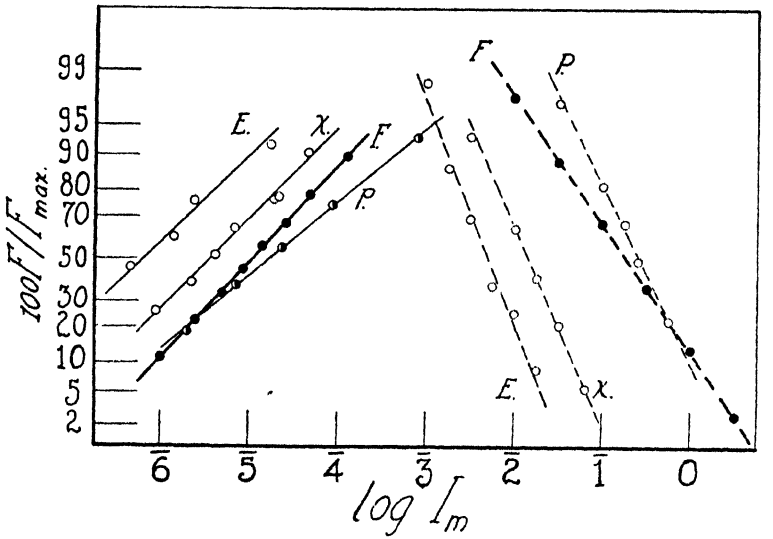

FIG. 4. The rising rod curves, F *vs.* log I_m, for the teleosts considered in Fig. 3 are shown to be well described by probability integrals. The comparatively large size of the *Fundulus* curve (*F.*) permits a more precise test than with the curves of other fishes. The declining curves, at the right, are the difference curves as in Fig. 2; the plotted points were read from these curves.

matter of some interest for interpretive ethology. Apparently, however, no such correlations obtain. This is consistent with the independent behavior of the two in a genetic experiment.[8]

IV

The variation of critical intensity has a number of important aspects.[24] As shown in Fig. 5, the scatter of I_1, measured by the dispersion of the ten mean values of I_e at each F and computed as P.E.$_{1_{I_1}}$, obeys precisely the

[23] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *d*, *e*.

[24] Crozier, 1935; 1935–36; 1936; Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *a*, *b*; 1937–38 *a*.

rules found with other vertebrates.[1, 2, 3, 24] P.E.$_{I_1}$ is directly proportional to I_m, up to a rather high intensity at which the graph assumes a lower slope. The portion of slope < 1 has been interpreted as one of direct pro-

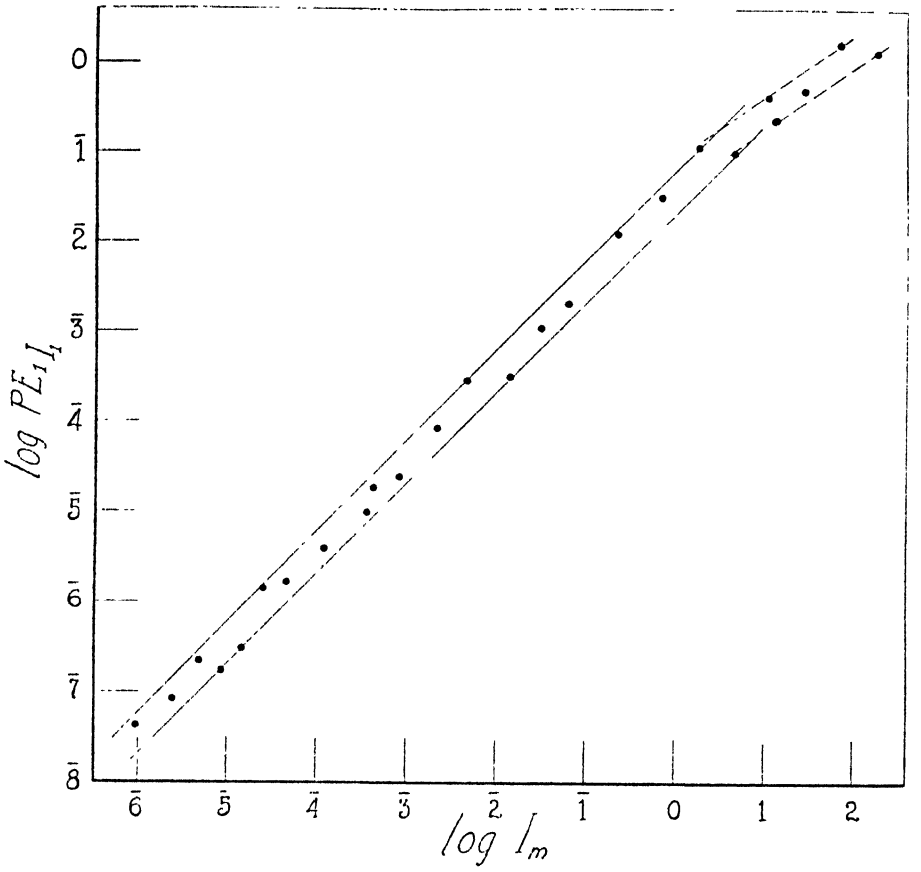

FIG. 5. The variation (σ_{I_1}) of critical intensity (I_1) for response to flicker (Table I) is a rectilinear function of I_m, with a slope of 1. Beyond a certain intensity (log I_m = 0.5) a new origin must be taken, so that the plot "breaks" at this point. This is the intensity at which the declining rod curve approaches the log I axis (Fig. 2). Above this intensity only cone effects are concerned.

portion to I_m but with a new origin.[24] The break has been correlated with the disappearance of rod contributions to the determination of the response.[24] It will be observed that the break occurs with *Fundulus* at log I_m = 0.5. This is precisely the level of intensity at which (Fig. 3) the declining rod contribution curve is required to fuse asymptotically with the abscissa axis. This constitutes an independent test of the analysis.

Table II contains a summary of the corresponding facts for other animals. The correlation is precise, as in each instance, the break in the plot of log P.E.$_{I_1}$ comes exactly a little above the disappearance of imputed rod effect on the composite F-log I curve. This simply cannot be a matter of error due to the use of the instrument producing flicker, which was the same in all cases. Three animals which do not show retinal duplexity,

TABLE II

Values of log I_m at which a break occurs in the graph of σ_{I_1} *vs.* log I_m in the flicker response data of various animals.[3] In each case there is correspondence with the intensity beyond which cone-originating effects are exclusively involved in the determination of the critical illumination for response, as shown by the suppression of the contribution from rods required to give a quantitative dissection of the composite graph. With three forms (bee; *Anax* larva; the turtle *Pseudemys*) there is involved only *one* class of retinal elements, and no break in the σ_{I_1} *vs.* log I_m curve is detectable although the range of intensities covered is the same for all forms. Thus the break cannot be attributed to "experimental errors" and their rôle in contributing to σ_I, but is a specific resultant of the fact that the measured variation of critical intensity is primarily an index of the variability of the reacting organism.

Animal	log I at the break
Fundulus	0.5
Platypoecilius	0.0
Xiphophorus	$\bar{1}.0$
Hybrid of *X.* and *P.*	0.0–
Enneacanthus	$\bar{1}.0$
Rana	$\bar{3}.5$
Triturus	0.3
Man	$\bar{2}.7$; $\bar{2}.8$
Apis, *Anax* larva, *Pseudemys*, Gecko	None

Anax, *Pseudemys*, and *Sphaerodactylus*, show no break in the graphs of log P.E.$_{I_1}$ *vs.* log I_m, although covering the whole range of measured intensities. These facts are obviously consistent with the view that the variation measured is a property of the reacting organisms,[24, 25] and is not an expression of manipulative "error."

V

The retina of *Fundulus* has been described as exhibiting a regional differentiation into a dorsal part (70 per cent of the total) containing rods and

[25] Crozier and Holway, 1938.

two types of cones (one type with an oil globule), and a ventral portion with rods and one type of cone (small, and lacking oil globule).[26] The rods are about twice as numerous as the cones. This might correspond to the large share taken by the rod function in the composite flicker contour (Fig. 2), but for the fact that data on *Rana* and other forms show that no quantitative ratio of this kind can be relied upon. So far as the data on reaction to flashes go, the several kinds of cones described by Butcher[26] form parts of a unified population. If, as Butcher's observations suggest, the dorsal cones with oil globule are concerned with chromatophore response to backgrounds, they, of course, might not be chiefly implicated in flicker effects at all. The horizontal direction of the light responsible for flashes in our experiments should result in equivalent illumination of dorsal and ventral halves of the retina.

The temptation is always great to stress one-to-one correlations between rods and cones and duplexity of visual function. The correlation has pitfalls. The effects measured are not immediately retinal. The presence of two chief kinds of retinal sensory cells is suggestive in connection with a double F-log I curve, particularly when animals with no rods or no cones have a simple curve. But it cannot be assumed that the presence of two histological kinds of cones will necessarily signify a further subdivision of the classes of central nervous elements responsible for a given particular type of response.

VI

When the F-log I_m curve for a given animal is determined at various temperatures of the organism it is found that the maximum to which the curve rises is not changed. For each change of temperature the curve is simply moved to a different position on the log I axis, without change of shape.[27] The shift is such that for a vertebrate whose curve exhibits two distinct sections the temperature characteristics for change in position are the same for the two parts. For fixed F we find that $1/I_m$ is related to temperature, within the range where free reversibility of effect is obtainable, by the Arrhenius equation;[28] hence (with the support of other relevant considerations) we may assume that $1/I_m$ behaves as if controlled by the velocity of an underlying system of chemical changes in which one or another catalytic link may be the pace-making step.

The question thus arises as to whether it is possible to decide if the

[26] Butcher, 1938.

[27] Crozier, Wolf, and Zerrahn-Wolf, 1936–37 *c*, *d*; 1938–39; Crozier and Wolf, 1938–39 *c*.

chemical change appealed to is directly responsible for the obedience of $1/I_m$ to the Arrhenius equation, or whether it does so by secondary control of the number of effective available units or neural elements. Since $F_{max.}$ is not affected,[27, 28] it must be decided that the total number of available neural elements is not the factor influenced by temperature.

It is well known that by exposing certain organisms to sufficiently high temperatures, above a "critical temperature" (or above the mislabelled "optimum" in ordinary experiments) for a sufficient time, one may produce changes which are only slowly reversible ("injury" and the like). If such changes are produced in the flicker response contour of a vertebrate, we may obtain information as to whether (1) the total number of neural elements available for discrimination of flicker has then been affected, or (2) whether a chemical system common to all these elements has been altered in a persistent way; and thus (3) whether this system is different in the rod and cone populations of elements. We already know[28] that the differing shapes of the rod and cone segments of the F-log I contour cannot be regarded as signifying the operation of chemically different systems in the respective elements. The effect of a durable modification of the F-log I curve by heat treatment shows that the "rod" and "cone" elements in *Fundulus* cannot be differentiated by this means.

For such an experiment *Fundulus* has peculiar advantages. Its F-log I curve is exceptional in the large contribution made by presumptive rod-excited elements. It is known that with *Fundulus* by careful adaptation to supranormal temperatures persisting physiological alterations may be induced.[29] We find that by exposure of winter-caught *F. heteroclitus* to 25–27° for 4 days the F-log I curve of individuals then kept for a week or longer at 21.5° has been "permanently" shifted in the direction of lower intensities, so that, although measured at 21.5°, its position relative to that for the unheated individuals is still the one expected for normal animals at 25–27°.

As Fig. 6 demonstrates, the induced shift in the curve shows no change in $F_{max.}$, and no change in shape. The rod part is displaced to the same extent as the cone, and its maximum is likewise unaffected. Therefore it cannot be maintained that the number of neural elements available for recognition of flicker has been affected. Since this number is clearly a function of peripheral sensory area involved,[30] and is also appropriately

[28] Crozier, 1939; Crozier and Wolf, 1939 *a*, *b*; 1939–40 *a*.

[29] Loeb, 1913; Loeb and Wasteneys, 1912; Crozier, 1919.

[30] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*.

related to the proportion of light time in a flash cycle,[31] it must be admitted to be a sensitive index. The persisting change resulting from exposure to supranormal temperature must be conceived as governed by an enduring change in the chemical system regulating the excitability of all these

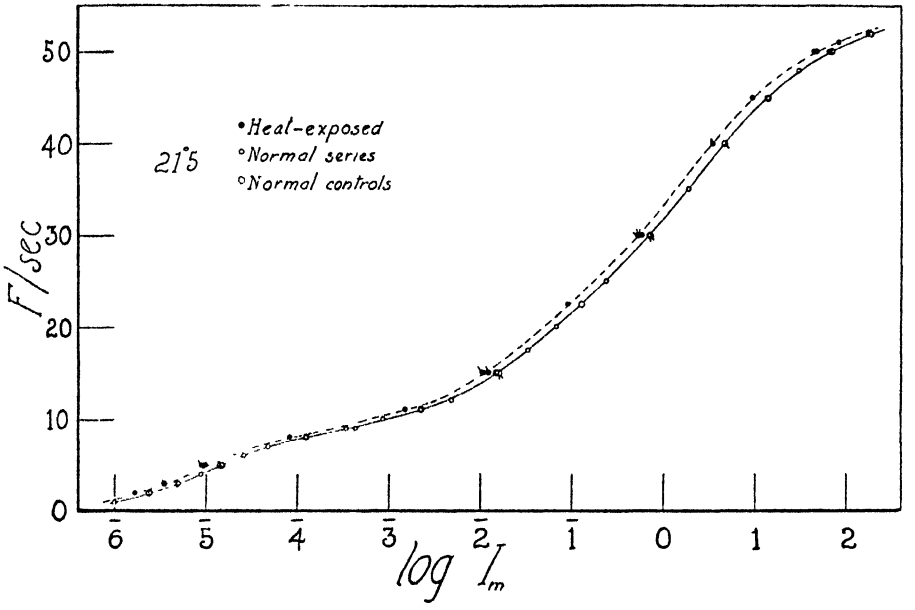

FIG. 6. Mean critical intensity (I_m) as a function of flash frequency for *Fundulus heteroclitus;* means of three observations on each of eight individuals at all points, at 21.5° C., with a flash cycle having equal durations of light and darkness. The "heat-exposed" series, measured after exposure to 25–27° for 4 days; the "normal series," and the "normal controls," were not exposed to temperatures above that of the room. Duplications of points (shown by tags when overlapping) are based on repetitions of the experiment with another lot of ten individuals. The same curve is drawn for both sets of measurements, with displacement by 0.16 log units to the left for the heat-exposed series.

neural elements. The analysis of duplex flicker contours has shown that in the region of overlapping of rod and cone effects there is involved a declining rod contribution; the *Fundulus* curve is important because of the prominence of this feature, and the absence of any change in the form of the curve for the heat-exposed individuals shows that the "decay curve" for the presumptive rod contribution is likewise unaffected as to its shape.

[31] Crozier, Wolf, and Zerrahn-Wolf, 1937–38 *c*, *d*.

The *variability*[32] of critical intensity is precisely the same function of the intensity as with normal *Fundulus* (Fig. 7).

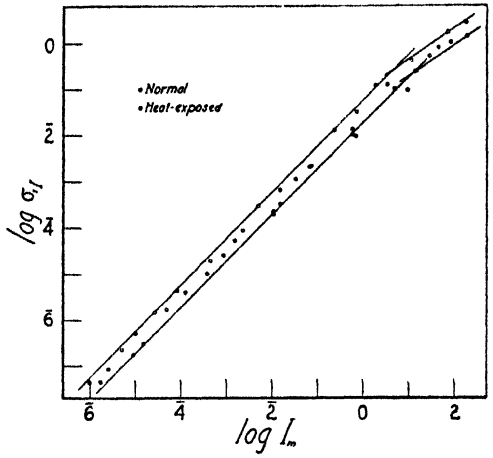

FIG. 7. The dispersion of the individual determinations of I_1, as a function of I_m, is identical for the normal and the heat-exposed series of *Fundulus*. Up to I_m = antilog 0.5, the variation and the mean are directly proportional.

The shift in the curve cannot be explained by enlargement of the iris aperture, as result of the exposure, since direct observation of these animals in a thermostat shows that no measurable change is produced. In the intact fish the iris diameter is independent of temperature (12–28°) and of illumination. Moreover, enlargement of the iris might not raise the curve vertically, nor shift it to the left (Fig. 6). It is not reasonable to suppose that the ocular media become persistently more transparent with heat treatment; any effect is more likely to be in the direction of increased opacity, if anything. Most of our heat-treated individuals died in about 2 weeks after return to room temperature; one was alive after 4 weeks. After a week at 21°, critical intensity determinations with previously heated *Fundulus* slowly drifted after 10 to 14 days up to the levels obtained before exposure to 27°, showing that the effect is reversible.

VII

SUMMARY

After *Fundulus heteroclitus* have been for some time in the laboratory, under conditions favorable for growth, and after habituation of the fishes to the simple routine manipulations of the observational procedure required, they are found to give reproducible values of the mean critical flash illumination (I_m) resulting in response to visual flicker. The measurements were made with equality of light time and dark time in the flash cycle, at 21.5°C.

Log I_m as a function of flash frequency F has the same general form as

[32] Crozier, 1936; 1935–36; Upton and Crozier, 1936; Crozier and Holway, 1937; 1938–39.

that obtained with other fishes tested, and for vertebrates typically: the curve is a drawn-out S, with a second inflection at the low I end.

In details, however, the curve is somewhat extreme. Its composite form is readily resolved into the two usual parts. Each of these expresses a contribution in which log I, as a function of F, is accurately expressed by taking F as the summation (integral) of a probability distribution of d log I, as for the flicker response contour of other animals.

As critical intensity I increases, the contribution of rod elements gradually fades out; this decay also adheres to a probability integral.

The rod contribution seen in the curve for *Fundulus* is larger, absolutely and relatively to that from the cones, than that found with a number of other vertebrates. The additive overlapping of the rod and cone effects therefore produces a comparatively extreme distortion of the resulting F-log I curve.

The F-log I_m curve is shifted to lower intensities as result of previous exposure to supranormal temperatures. This effect is only very slowly reversible. The value of $F_{max.}$ for each of the components of the duplex curve remains unaffected. The rod and cone segments are shifted to the same extent. The persisting increase of excitability thus fails to reveal any chemical or other differentiation of the excitability mechanism in the two groups of elements.

Certain bearings of the data upon the theory of the flicker response contour are discussed, with reference to the measurements of variation of critical intensity and to the form of the F-log I curve. The quantitative properties of the data accord with the theory derived from earlier observations on other forms.

We again wish to thank Dr. Gertrud Zerrahn-Wolf for her collaboration in the observations.

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THE PURIFICATION OF CATHEPSIN

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The Cathepsin System.—Extracts of animal tissues contain a proteolytic system which is active at pH 3.5. This system consists of a proteinase part called "cathepsin" which initiates the digestion of protein, and a peptidase part which carries the digestion further. The activity of the tissue peptidase can be increased by cysteine and abolished by iodoacetic acid. The activity of the tissue proteinase, however, is not affected by these reagents (Anson, 1937). To estimate the proteinase alone denatured hemoglobin is digested for a short time and to a slight extent at pH 3.5, the undigested protein is precipitated by *dilute* (0.19 N) trichloracetic acid, and the unprecipitated digestion products are estimated by the color they give with the phenol reagent (Anson, 1937, 1938).

Fruton and Bergmann (1939) have now found synthetic peptide substrates for two different tissue enzymes. One of these enzymes is activated by cysteine, the other is not.

The Function of Cathepsin. --Cathepsin is the only proteinase known to be present in animal cells and unicellular organisms generally. Those cells which are particularly active in the synthesis of proteins are particularly rich in cathepsin. The optimum pH for the digestion of proteins by tissue cathepsin is 3.5, whereas living tissues are much more alkaline. There is, at present, no direct evidence concerning the function of cathepsin in living cells. The most likely hypothesis is that cathepsin catalyzes the synthesis of cellular proteins.

Crude extracts of animal tissue which contain a mixture of proteolytic enzymes carry the digestion of protein very far. It would hardly be expected that such extensive breakdown could be reversed *in vitro*. Thus, the first step in experiments on *in vitro* synthesis of protein is the preparation of cathepsin as free from other proteolytic enzymes as possible.

The Purification of Cathepsin.—This paper describes the purification of cathepsin extracted from beef spleen. 1 mg. of the protein in the purified preparation has the same activity as the extract of 1.3 gm. of spleen. Some

inert protein is first destroyed by autolysis. More inert protein is removed by adsorption first by spleen material and then by aluminum hydroxide. Finally, the cathepsin and most of the remaining inert protein is precipitated from a very dilute solution by tungstic acid. The tungstic acid precipitate of cathepsin is stable in the cold.

The specific activity of the protein precipitated by tungstic acid can be increased at least 8 times by extraction of the tungstic acid precipitate with barium hydroxide plus barium chloride and by the application to this extract of further procedures which have not yet been standardized and so will not be described in this paper.

Purified cathepsin is not inactivated when exposed to 0.01 M iodoacetamide for an hour at pH 8.0 and at 25°C. When added to gelatin at pH 3.5 in the presence of cysteine it does not cause a detectable increase in the formol titration. The experiments with gelatin were not carried out with the cathepsin preparation described in this paper.

The Starting Material.—Beef spleen is used as a source of cathepsin because it is relatively rich in cathepsin and relatively cheap. Even spleen, however, contains extremely little proteinase compared with specialized tissues such as exist in the stomach and pancreas. Thus, the extract of 1 gm. of beef spleen has 3.7×10^{-3} hemoglobin units of cathepsin activity whereas the extract of 1 gm. of pig stomach fundus mucosa has 5×10^{-1} hemoglobin units of pepsin activity.

The Purification Procedure.—The following outline of the procedure used for the purification of cathepsin states in a general way what the various steps are, what they accomplish, and in what way they are of general interest for the methodology of the purification of tissue proteins. Detailed directions are given in the experimental part.

1. Frozen beef spleen is thawed and suspended in water for a day. During this procedure some autolysis takes place. About half the nitrogen of the spleen is extracted and about half the nitrogen in the extract is protein nitrogen.

2. The suspension is 0.3 saturated with ammonium sulfate, acidified to green to brom cresol green, and heated to 45°C. Extensive further autolysis takes place, denatured protein is precipitated, and the mass of insoluble material is clotted, which facilitates filtration. The bulky mass of insoluble material adsorbs the cathepsin as well as practically all the unautolyzed other protein present. When the acidified and heated spleen material is filtered most of the products of autolysis remain in the filtrate and are thus removed.

Cathepsin not adsorbed to insoluble spleen material is unstable at 45°C.

in acid solution. Thus the adsorption protects the cathepsin from destruction under the conditions which bring about extensive autolysis. Purified cathepsin not adsorbed to insoluble spleen material is soluble in 0.3 saturated ammonium sulfate. Thus the adsorption also makes possible the separation of cathepsin from split products formed during autolysis.

3. The insoluble mass remaining after filtration of the acid autolysate is made slightly alkaline (red to phenol red) with a sodium hydroxide solution of the amphoteric aluminum hydroxide. The aluminum hydroxide which is soluble only in strong alkali is precipitated again at red to phenol red and helps with the filtration. It is not present in sufficiently large amount to be important as an adsorbent. The insoluble spleen material which adsorbed all the cathepsin in the acid ammonium sulfate solution no longer adsorbs cathepsin at red to phenol red and so after filtration the cathepsin is all obtained in the filtrate. Some inert protein, however, remains adsorbed to the insoluble spleen material and thus a purification as well as an extraction of the cathepsin is obtained by the addition of alkali. If the suspension is made too alkaline less inert protein is removed. If the solution is made too acid some cathepsin is carried down with the precipitate.

4. Some inert protein in the alkaline filtrate and about 25 per cent of the cathepsin are removed by adsorption with aluminum hydroxide at red to phenol red. If more than the recommended aluminum hydroxide is used or the pH is made less alkaline, more cathepsin is lost. If the amount of aluminum hydroxide is decreased or the pH made more alkaline, less inert protein is removed and a product of lower specific activity obtained. This procedure, like most adsorptions by hydroxide gels, is sensitive to the exact conditions and so does not give completely reproducible results.

Instead of adding previously prepared aluminum hydroxide, as is usually done, aluminum hydroxide in the procedure just described is formed in the spleen extract by the addition first of partially neutralized aluminum chloride and then of sodium hydroxide. Aluminum hydroxide formed in the extract removes much more inert protein than the same amount of aluminum hydroxide prepared before being added to the extract. Presumably protein does not penetrate rapidly into the interior of an aluminum hydroxide gel.

5. The cathepsin and most of the remaining protein and a part of the protein split products are precipitated by tungstic acid at green to brom cresol green. At this pH the proteins are precipitated by tungstic acid in the native form. When tungstic acid is used to remove proteins for analyti-

cal work on protein-free filtrates, the protein is usually denatured as the result of the solution being made too acid. So tungstic acid has in general not been used for preparative work. Tungstic acid is chosen for the precipitation of cathepsin despite the fact that it precipitates some of the protein split products. It is the only reagent I have found which will precipitate cathepsin even when the cathepsin is present in an extremely dilute solution containing much more protein split products than protein and which is cheap enough to be used for very large volumes of solutions. The presence of tungstate, furthermore, is useful in the further purification of cathepsin with barium hydroxide.

TABLE I

Purification of Cathepsin

	Hb units per gm. spleen	Hb units per mg. protein N	Hb units per mg. tyrosine protein color value	Hb units per mg. tyrosine total color value
	$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$
Filtrate from autolyzed spleen suspension	37	4.6	11.8	6.5
Alkaline extract of acidified and heated spleen	18		94	17.5
Alkaline extract purified with $Al(OH)_3$	13.5	114	330	22
Tungstic acid precipitate of purified extract	12			120

Many techniques which are used in the fractionation of proteins are not practical for large scale work with tissues. Yet if many experiments are to be done on the purification of an enzyme which is present in low concentrations large scale work is necessary. The procedure for the purification of cathepsin which has been outlined permits one man to work up as much as 40 kilograms of spleen at a time with very simple apparatus and can also be applied to small quantities of spleen.

Results.—Table I gives a typical series of results. The first column shows the amount of proteinase left at each step per gram of spleen starting material. About half the loss of proteinase is a purely mechanical loss due to the fact that in the alkaline extraction the precipitate is not washed. The other columns give the activities per milligram protein nitrogen and per unit total and protein color value. By protein is meant the material precipitated by 0.2 N trichloracetic acid. The protein content of the tungstic acid precipitate is not given because protein cannot be estimated in the presence of tungstic acid which precipitates protein split products.

The color value is the color given with the phenol reagent expressed as the number of milligrams of tyrosine which give the same color. The color value is easier to measure than the nitrogen value and it can be measured in the presence of ammonium sulfate. Purification of the cathepsin changes the protein color value per milligram of protein nitrogen only slightly. Although activity, nitrogen, and color values are given for the filtrate from the suspension of thawed spleen, in practice this suspension is not filtered but is acidified and heated.

Note on Maver's Experiments.—Maver (1939) digested denatured hemoglobin at pH 3.5 for 3 hours at 37°C. with a somewhat purified liver extract and measured by the Kjeldahl method the amount of protein precipitated by trichloracetic acid both before and after digestion. She found that the amount of protein changed into a non-precipitable form was increased 3.8 times by the addition of cysteine and concluded that the proteinase, cathepsin, is activated by cysteine. The experimental conditions used by Maver differ in two important respects from the conditions used in my hemoglobin method. The trichloracetic acid is 1 N instead of 0.187 N and the digestion is carried on much longer and much further than in my original experiments.

The conditions used by Maver, apart from being inconvenient, are not suitable for the estimation of proteinase in the presence of peptidase. I have found that for a given amount of digestion the nitrogen content of the trichloracetic acid filtrate of a hemoglobin digest is 1.5–3.0 times less when the trichloracetic acid concentration is 1 N than when it is 0.2 N. The exact difference depends on the extent and conditions of digestion.[1] Hiller and Van Slyke (1922) showed that 10 per cent trichloracetic acid precipitates somewhat more of Witte's peptone than 2.5 per cent trichloracetic acid. Thus, under Maver's conditions one estimates not only the initial digestion of protein due to proteinase but also the further digestion of split products precipitable by concentrated but not by dilute trichloracetic acid which may be due to peptidase. Secondly, in the estimation of proteinase it is not desirable to carry the digestion far since extensive digestion introduces complications due to the inhibition of proteinase by the products of digestion. When the extent of digestion of protein is great the extent of digestion is no longer sensitive to the amount of enzyme used and an apparent activation of proteinase may really be an activation of peptidases which digest split product inhibitors.

I have digested hemoglobin for 3 hours with a crude spleen extract, added trichloracetic acid, filtered off the precipitate, and estimated by the Kjeldahl method the nitrogen

[1] This observation suggests a method for preparing large split products from proteins. If the protein is digested *slightly* and the undigested protein is precipitated by 0.2 N trichloracetic acid and removed by filtration, then most of the material in the filtrate can be precipitated by the addition of more trichloracetic acid. The exact concentration of trichloracetic acid for maximum precipitation should be determined empirically, since the amount precipitated decreases again if the trichloracetic acid is made too concentrated. 1 N trichloracetic acid was used in the present experiments only because it was used by Maver. As digestion proceeds the fraction of the material in the filtrate precipitable by the addition of more trichloracetic acid becomes less.

content of the filtrate. When a small amount of enzyme was used and the final concentration of trichloracetic acid was 0.2 N then the nitrogen content of the filtrate (corrected for the blank) was 1.22 times as great when cysteine was present during the digestion than when cysteine was not added. When, however, 12 times as much enzyme was used and the final trichloracetic acid concentration was 1 N then the filtrate contained 1.54 times more nitrogen if cysteine was present than if it was not (Table II).

I do not know the reason for the small and unimportant cysteine effect obtained even when the extent of digestion is small and dilute trichloracetic acid is used. There may be some inactivation of cathepsin in the 3 hour digestion period used by Maver, which inactivation is inhibited by cysteine. I have already shown that such inactivation and inhibition can take place in more acid solution (Anson, 1937). There may be some small salt effect such as can also be obtained with ammonium sulfate. In any case, there is no evidence that the small cysteine effect is due to activation of enzyme at all. Enzymes of the papain type are usually almost completely inactivated by much handling and the cysteine effect is usually very great.

TABLE II

Effect of Cysteine on Digestion of 100 Mg. Hemoglobin

Amount crude enzyme used for digestion mg. N	Presence of cysteine	Final normality of trichloracetic acid	Mg. N in total 17 cc. trichloracetic acid filtrate—corrected for blank
0.068	−	0.2	0.86
0.068	+	0.2	1.04
12 × 0.068	−	1.0	2.7
12 × 0.068	+	1.0	4.0

The somewhat larger cysteine effect obtained when, following Maver, extensive digestion and concentrated trichloracetic acid are used is probably due to an activation of peptidase. Tissue peptidase is activated by cysteine (Anson, 1937) and, as has already been pointed out, under Maver's conditions the hemoglobin-trichloracetic acid method may well measure peptidase in addition to proteinase.

The present experiments do not explain why Maver observed a 280 per cent increase in the nitrogen content of the trichloracetic acid filtrate when I observed only a 54 per cent increase. The two enzyme preparations were different and perhaps Maver's enzyme preparation did contain, as she concluded, a proteinase activated by cysteine.

In conclusion, when conditions are chosen under which proteinase alone is measured, no large cysteine effect is observed even when a long digestion period is used and the split products are estimated by the Kjeldahl method. The large cysteine effect reported by Maver cannot be accepted as an activation of proteinase until it is shown that under Maver's conditions only proteinase is estimated.

EXPERIMENTAL

Estimation of Cathepsin.—Cathepsin is estimated as previously described (Anson, 1938). The preparation of the hemoglobin substrate has, however, been modified by the treatment of the red blood corpuscles with toluol (Anson, 1939). It is necessary that the empirical directions be followed rigorously if it is desired to express the activities

measured in terms of the standard hemoglobin units. The following precautions should be taken in preparing the reagents and carrying out the procedure.

The first steps in preparing the hemoglobin substrate consist in centrifuging whipped blood, washing the red corpuscles with cold salt solution, and shaking the washed corpuscles with toluol. These steps should be carried out the same day the blood is obtained or, if this is not possible, the blood should be promptly cooled to 0°C., kept at 0°C., and worked up as soon as possible. If much bacterial multiplication takes place before the addition of toluol then split products are formed which are not completely removed by dialysis and, in addition, the substrate solution becomes contaminated with bacterial enzymes.

After the corpuscles have been shaken with toluol and the suspension is filtered, if there is a layer of toluol over the filtrate the filtrate is allowed to stand in a cylinder and the toluol is removed with a fine tipped syphon. Large amounts of toluol are not completely removed by the subsequent dialysis and toluol decreases the rate of digestion by cathepsin.

The dialyzed hemoglobin solution is stored frozen in cardboard or aluminum containers. These containers should be moisture tight so that the concentration of hemoglobin does not change with time. Wax paper should be placed between the covers of the containers and the containers and the joints on the bottom of the usual cardboard containers should be covered with paraffin.

When the hemoglobin solution is thawed for use only such an amount should be thawed as will be used in a week or two. This thawed hemoglobin solution is stored at 0–5°C. Eventually bacterial growth sets in. I have found no effective preservative which does not interfere with either the digestion or the colorimetric estimation of the products of digestion.

The dialyzed hemoglobin solution still contains a small amount of substances not precipitable with trichloracetic acid which give a color with the phenol reagent. This blank is measured from time to time by the method already described. If, as a result of bacterial action, the blank is high or rising the hemoglobin should be rejected rather than any attempt made to correct for the blank. The blank of the hemoglobin substrate prepared by the present procedure changes only slightly with storage of the hemoglobin until bacterial action finally sets in in the thawed solution.

After the hemoglobin has been partially digested by cathepsin the undigested hemoglobin is precipitated by the addition of 0.3 N trichloracetic acid, the suspension is filtered, and the filtrate is made alkaline with 0.5 N sodium hydroxide. It is important that the concentrations of trichloracetic acid and sodium hydroxide be checked by titration. Commercial solid trichloracetic acid contains varying amounts of water. A filter paper such as Whatman's No. 42 should be used which gives a clear filtrate and does not adsorb split products. The color value of the filtrate, as measured with the phenol reagent, should be the same as that of the supernatant solution obtained if the trichloracetic acid suspension is centrifuged.

To check the whole procedure the hemoglobin is digested with a standard cathepsin solution and the products of digestion estimated. A stock stable solution of cathepsin in 75 per cent glycerine is stored at 5°C. 1 cc. of this glycerine solution is removed with an Ostwald contain pipette and diluted to 15 cc. with water to give the standard solution used for digestion. Different batches of hemoglobin are digested at the same rate by a given amount of cathepsin and the hemoglobin can be stored frozen for 6 months without any effect on the rate at which it is digested.

Estimation of Protein.—The protein is precipitated by 0.2 N trichloracetic acid out of a solution diluted to contain about 0.3 mg. protein per cc. When the solution contains much protein split products it is necessary to leave the trichloracetic acid solution at the temperature of hot tap water for a few minutes and then cool it to room temperature. If the heating is omitted the precipitation of protein is very slow. If the cooling to room temperature is omitted some of the protein remains dissolved.

To estimate the protein colorimetrically, a trichloracetic acid precipitate containing about 3 mg. of protein is dissolved with 8 cc. of 0.5 N sodium hydroxide and 7 cc. of water. The color is then developed with 3 cc. of diluted phenol reagent as in the estimation of cathepsin (Anson, 1938). In the cathepsin procedure more sodium hydroxide is used because of the trichloracetic acid which has to be neutralized.

Purification Procedure.—Beef spleen is ground and frozen in one pound blocks at the slaughter house (Swift and Co., Chicago) and transported and stored frozen.

The frozen spleen is allowed to thaw in a cold room and then suspended in twice its weight of tap water and allowed to stand for at least 24 hours at room temperature with toluol (Eastman Practical) as a preservative. For small scale work the spleen is suspended in an aluminum milk pail (Sears Roebuck). For large scale work the spleen suspension is prepared in 60 liter aluminum kettles (Wear-Ever Semi-Heavy Stock Pot).

To each liter of suspension there are added 176 gm. of ammonium sulfate and, after the salt is dissolved, 70 cc. of 1 N HCl. During the addition of the acid the suspension is stirred vigorously by hand with a large wooden paddle such as can be obtained from hotel supply houses. Mechanical stirring results in hard packing of the insoluble spleen material.

The spleen suspension is heated to 45°C. with the aid of an aluminum coil through which 50–55°C. water is passed. A thermometer is introduced into the system with a Y-shaped aluminum connecting tube (Fischer Scientific Co., No. 15-321B) the thermometer being attached with thick rubber tubing. The suspension is stirred with the wooden paddle during the heating. It takes about 20 minutes to heat 35–40 liters of suspension with a 40 foot coil. The large kettle is covered with an aluminum cover and with blankets to prevent rapid cooling and is allowed to stand overnight. When a small amount of suspension is used it is transferred to a bottle which can be closed and is put away at 37°C.

The next morning the suspension is filtered on 50 cm. folded filter paper, the No. 612 sold in unfolded form by Eaton Dikerman Company, Mount Holly Springs, Pennsylvania. More suspension is poured on the papers when there is room, the final amount of suspension added to each funnel being about one and one-half times the amount needed to fill the funnel originally. After the filtration is about complete but before the solid material has begun to crack, the funnels are filled with tap water made green to brom cresol green with HCl, covered with aluminum covers (Sears Roebuck), and the filtration is allowed to continue overnight. More extensive washing is not possible without loss of cathepsin.

The next morning, the precipitates and the filter papers are stirred up with tap water, the total volume being that of the original spleen suspension before the addition of salt and acid. More toluol is added. The suspension is brought to red to phenol red by adding with stirring a solution made up of 1 part 2 M $AlCl_3$ and 10 parts 1 N NaOH. The suspension is stirred vigorously and more alkali is added as needed to keep the suspension red to phenol red.

After the suspension has been at red to phenol red for 1 hour it is poured on the large folded filter papers, covered, and allowed to filter overnight, no additional suspension being added to each paper. The precipitate is not washed although it occupies one third of the original volume. Washing is slow and results in a product with lower specific activity.

A partially neutralized $AlCl_3$ is made up by adding 1 N NaOH with mechanical stirring to an equal volume of 1 M $AlCl_3$. 40 cc. of this product are added to each liter of alkaline filtrate. 1 N NaOH is then added with mechanical stirring until the solution is red to phenol red and the $Al(OH)_3$ is filtered off with the aid of Hyflo Super-cel (Johns Manville) and suction on monel metal Buchner funnels of 33 cm. diameter (Louis Fuhro, New York City). The filter cake is washed with water made red to phenol red with NaOH until the total volume of the filtrate is about the same as the suspension before filtration.

Since the exact amount of $Al(OH)_3$ which gives considerable purification without too great loss of cathepsin is somewhat variable, it is safest, in large scale work, to find out by preliminary experiments with samples how much $Al(OH)_3$ gives about 25 per cent loss of cathepsin.

To each liter of filtrate, which must be at room temperature and not cold, there are added 10 cc. of 1 M technical sodium tungstate (Molybdenum Corporation of America) and with mechanical stirring enough 1 N HCl to make the solution green to brom cresol green. The suspension is allowed to stand 1 hour with occasional stirring. More HCl is added if necessary to keep the solution green to brom cresol green. The precipitate is filtered on large Buchner funnels with Hyflo Super-cel and is washed with 0.001 M tungstate made green to brom cresol green until the wash water gives the same sort of precipitate with a barium salt as the 0.001 M tungstic acid solution alone. When there is sulfate still present it is readily detected both by the amount and the character of the precipitate.

Finally the filter cake is suspended in water to give a suspension containing about 0.01 hemoglobin units of cathepsin per cc., assuming all the cathepsin to have been precipitated from the solution to which tungstic acid was added. Toluol is added as a preservative and the suspension is stored at 5°C. or frozen.

The Effect of Iodoacetamide on Cathepsin.—A tungstic acid precipitate of purified cathepsin is dissolved with sodium hydroxide. The final solution is red to phenol red and contains 2×10^{-4} hemoglobin units of cathepsin per cc. If the cathepsin is allowed to stand for 1 hour at 25°C. at red to phenol red in the presence of 0.01 M iodoacetamide no inactivation takes place.

The Effect of Cysteine on Digestion.—The hemoglobin substrate is made up without ammonium sulfate by adding 1 cc. of 1.35 M acetic acid to 4 cc. of 2.5 per cent dialyzed hemoglobin prepared as already described (Anson, 1938, 1939). To 5 cc. of this solution there is added either 0.5 cc. of 0.2 M cysteine HCl and 0.5 cc. of 0.16 N NaOH or in the control experiment 1 cc. of 0.1 N NaCl. 1 cc. of enzyme solution is then added and digestion is carried out for 3 hours at 37°C. 10 cc. of 0.34 N trichloracetic acid are added to give a final concentration of 0.2 N or 10 cc. of 1.7 N trichloracetic acid to give a final concentration of 1 N. The suspension is allowed to stand 2 hours before filtration. This may be a longer period than is needed to complete the precipitation but if the filtration of the concentrated trichloracetic acid suspension is carried out right away a precipitate gradually forms in the filtrate. The nitrogen content of 5 or 10 cc. filtrate is measured

by the Kjeldahl method. To measure the blank the substrate solution is left for 3 hours at 37°C. and the enzyme is added after the trichloracetic acid.

The enzyme is prepared by dialyzing a filtrate of the suspension of thawed spleen already described as the first step of the purification procedure. This crude preparation seems to be somewhat more active than the purified cathepsin used by Maver.

The results are shown in Table II.

SUMMARY

1. One mg. of the purified cathepsin whose preparation is described is as active as the extract of 1.3 gm. of spleen. An eightfold further purification is possible by procedures which are still being modified and so are not described in the present paper.

2. The first step of the purification consists in suspending frozen and thawed spleen in water and letting it autolyze.

3. In the second step, ammonium sulfate is added and the suspension is acidified and warmed. Much additional autolysis takes place. The cathepsin is protected from destruction by being adsorbed to the insoluble spleen material. When this insoluble material is filtered off most of the products of autolysis remain in the filtrate.

4. The cathepsin is then released from the insoluble spleen material by making the suspension slightly alkaline. Some inert protein still remains adsorbed to the insoluble spleen material.

5. More inert protein is removed by adsorption with aluminum hydroxide formed in the cathepsin solution by the addition first of aluminum chloride and then of sodium hydroxide. Preformed aluminum hydroxide is a much less effective adsorbent.

6. The purified cathepsin is precipitated from very dilute solution with tungstic acid. Tungstic acid precipitates most proteins in the native form provided the solution is not too acid.

7. Further evidence is given that cathepsin is not a proteinase of the papain type.

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THE PHOTODYNAMIC INACTIVATION OF PHAGE PRECURSOR BY METHYLENE BLUE*

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It has been shown that phage-susceptible staphylococci grown under optimal conditions in oxygenated broth and subsequently brought to the resting state by storage in Locke's solution at 5° C., have the property of rapidly producing a marked increase in [phage] upon addition to phage (1, 2). This effect, confirmed by Northrop (3), has been ascribed to the presence in the activated staphylococci of a phage precursor which when brought into contact with phage is autocatalytically transformed into phage (4). The intracellular precursor is more labile than either the cells which produce it or the phage into which it is converted (5, 6). For example, activated staphylococci can be made to lose their phage-augmenting capacity by the following procedures: (1) treatment with anti-staphylococcal serum; (2) heating at 45° C. for 20 minutes; (3) exposure to 1×10^{-4} M iodoacetic acid for 30 minutes. In all three cases the phage precursor content of the cells is destroyed before any measurable number of cell deaths has occurred as determined by plate counts. The data we present here indicate that similar inactivation of intracellular precursor can be obtained through the photodynamic action of methylene blue.

Suspensions of activated staphylococci were prepared as described in an earlier paper (4). The organisms were concentrated by centrifugation and were resuspended in Locke's solution of pH 7.0 to give a density of 1×10^{10} bacteria/ml. 1.0 ml. of activated cell suspension was added to 1.0 ml. of chemically pure methylene blue of appropriate concentration dissolved in Locke's solution. The mixtures were exposed to strong light of 4000–8000 Å emanating from a 500 watt cold crash lamp, 120 volts, type C, at a distance of 36 cm. from the end of the filament. During exposure the preparations were maintained at 5° C. and at intervals samples were removed for determination of viable cell content and for detection of residual phage precursor. For the latter purpose aliquots were diluted to contain 5×10^8 bacteria/ml. and 4.0 ml. of each sample were mixed with 1.0 ml. of phage containing 1×10^9 activity units/ml. The phage-cell mixtures were kept at 5° C. for 10 minutes to permit interaction of the residual precursor

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and the added phage. After this interval dilutions were made for titration by the activity method (7), using three dilution checks for each unknown.

Experimental controls included the following: (1) Duplicates of each mixture kept in the dark at 5° C.

(2) Experiments to detect:

(*a*) Any possible effect on the titration system of the small concentrations of methylene blue present in the final dilutions of each unknown.

(*b*) Lethal action of the concentrations of methylene blue used on activated staphylococci.

(*c*) Possible inactivating effect of methylene blue on phage during the 10 minute interval each mixture was kept.

(*d*) The effect of the light alone on the precursor content and viability of activated cells.

TABLE I

Photodynamic Inactivation of Intracellular Phage Precursor by Methylene Blue

Activated staphylococci in Locke's solution containing 9.1×10^{-7} M methylene blue exposed to strong light. Samples at intervals tested for residual phage precursor and for [bacteria]. Data represent averages of five consecutive experiments.

Molarity methylene blue	Time of exposure	[Phage] developed after adding exposed cells to phage $[Phage]_0$ of mixture = 2×10^8 P.U./ml.	[Bacteria] by plate count
	min.		
9.1×10^{-7} M	0 (Control)	1.22×10^9 P.U./ml.	7.0×10^9/ml.
9.1×10^{-7} M	1.0	1.5×10^9 P.U./ml.	6.2×10^9/ml.
9.1×10^{-7} M	5.0	1.4×10^9 P.U./ml.	6.2×10^9/ml.
9.1×10^{-7} M	10.0	1.32×10^9 P.U./ml.	6.4×10^9/ml.
9.1×10^{-7} M	15.0	1.25×10^9 P.U./ml.	5.6×10^9/ml.
9.1×10^{-7} M	30.0	4.8×10^8 P.U./ml.	5.8×10^9/ml.
9.1×10^{-7} M	45.0	3.6×10^8 P.U./ml.	7.2×10^9/ml.
9.1×10^{-7} M	60.0	2.2×10^8 P.U./ml.	5.7×10^9/ml.

The control experiments were completely negative; that is, the concentration of methylene blue employed had no direct action on phage, no killing action on the bacteria, no demonstrable effect on the titration system, nor did the mixtures kept in the dark show any inactivation of precursor. Further, exposure of activated cells to the light source did not result in precursor inactivation.

It is evident from the data summarized in Table I that 9.0×10^{-7} M methylene blue will inactivate the phage precursor in a suspension of activated staphylococci containing 5×10^9 bacteria/ml. exposed to light of 4000–8000 Å without producing cell death. With [bacteria] held constant this inactivating effect of methylene blue on phage precursor can be demon-

strated only over a very narrow range of methylene blue concentrations for relatively slight increases in [methylene blue] begin to cause cell death. If [methylene blue] is reduced appreciably the inactivating effect is lost.

1.0 ml. of 9.1×10^{-7} M methylene blue contains about 5×10^{14} molecules. The cell concentration used in the precursor inactivation experiments is 5×10^{9} bacteria/ml. Therefore there are available for each cell only 1×10^{5} molecules of dye assuming that all of it is taken up by the bacteria. If this ratio of 1×10^{5} molecules of methylene blue/bacterium is maintained when [bacteria] and [methylene blue] are varied, the photodynamic inactivation of phage precursor occurs over a fairly wide concentration range of reactants without causing cell death.

TABLE II

Experiments to Determine the Reaction Responsible for the Lag Phase of Photodynamic Inactivation of Phage Precursor by Methylene Blue

5×10^{9} activated bacteria/ml. in 9.1×10^{-7} M methylene blue kept in dark. After intervals noted, placed in light 5 min. and tested for [precursor] and [bacteria]			1×10^{10} activated bacteria/ml. exposed to light. At intervals 1 ml. samples mixed with 1 ml. methylene blue to 9.1×10^{-7}M. Kept in dark 5 min. and tested for [precursor] and [bacteria]		
Period exposed to methylene blue in dark	[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1×10^{9} units per ml.)	[Bacteria]	Period exposed to light (no methylene blue)	[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1×10^{9} units per ml.)	[Bacteria]
min.			*min.*		
5	2.4×10^{9}	5.6×10^{9}	10	2.1×10^{9}	6.4×10^{9}
15	2.6×10^{9}				
30	6.6×10^{8}	5.9×10^{9}	30	2.1×10^{9}	6.1×10^{9}
45	4.2×10^{8}				
60	2.5×10^{8}	4.6×10^{9}	60	1.7×10^{9}	6.8×10^{9}

It will be noted (Table I) that the first 15 minutes' exposure of activated bacteria to methylene blue in the light does not bring about any detectable reduction in the [precursor]. To determine the mechanism involved in this lag phase of the inactivation curve, activated cell suspensions were treated as follows:

1. A mixture containing 5×10^{9} activated bacteria per ml. and 9.1×10^{-7} M methylene blue was placed in the dark and samples were removed from it at short intervals. Each aliquot was placed in the light for 5 minutes, then diluted 1:10 in Locke's solution and tested for: (*a*) precursor content, and (*b*) [bacteria].

2. A suspension containing 1×10^{10} activated bacteria/ml. was exposed to the light source for periods up to 1 hour. 1 ml. samples were added to 1 ml. of 9.1×10^{-7} M methylene blue and the mixtures were kept in the dark

for 5 minutes before diluting 1:10 in Locke's solution and testing for: (*a*) precursor content, and (*b*) [bacteria].

The experimental results (Table II) indicate that the lag phase represents time consumed in a reaction taking place between the cells and methylene blue. Whether the limiting factor is diffusion into the cell, or a combination of diffusion and chemical action, the presence or absence of light seems to have no influence on the rate. When the primary reaction between the activated cells and methylene blue has gone to completion a relatively brief exposure to light will result in complete inactivation of the intracellular phage precursor.

SUMMARY AND CONCLUSIONS

Methylene blue added to suspensions of activated staphylococci in amounts sufficient to furnish 1×10^5 molecules of dye/bacterium inactivates the phage precursor content of the cells without causing cell death when the mixtures are exposed to strong light of 4000–8000 Å. There is a lag phase of approximately 15 minutes in the photodynamic inactivation of phage precursor by methylene blue. This delay seems to be due to a primary reaction between the cell and methylene blue after the completion of which exposure to light brings about the inactivation of precursor quite promptly.

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THE SENSIBILITY OF THE NOCTURNAL LONG-EARED OWL IN THE SPECTRUM

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I

Nature of Problem

In terms of the duplicity theory (Schultze, 1866; von Kries, 1929; Parinaud, 1885) the vision of nocturnal owls is considered homologous with our own vision at low light intensities. In fact, one of the original lines of evidence for Schultze's formulation of the theory lay in the differences in retinal structure between night birds such as the owl and day birds such as the chicken. According to Schultze the retinas of nocturnal birds consist predominantly of rods, whereas those of diurnal birds are composed mainly of cones. This histological differentiation has been amply confirmed (Rochon-Duvigneaud, 1919; Verrier, 1939), even to the extent of finding a large preponderance of rods in the lateral fovea of owls.

Later physiological work has emphasized the homology of the owl's vision with our own scotopic function. Piper's (1905) measurements of the retinal potentials produced in owls' eyes by different parts of the spectrum show a maximum response whose spectral position agrees not only with that of the maximum spectral sensibility of our own eyes at low intensities (Koenig and Ritter, 1891) but with the maximum light absorbed by visual purple in the same spectrum (Trendelenburg, 1904).

This whole picture of the owl's vision was unwittingly called into question by the report of Vanderplank (1934) that the nocturnal tawny owl, *Strix aluco*, is not especially sensitive to radiation in the visible portion of the spectrum, but is mainly responsive to infrared radiation. Vanderplank estimates the spectral range of the owl as lying between 600 and 1500 mμ, though he supposes this bird to be sensitive even to the far infrared.

The evidence presented by Vanderplank is direct and indirect. The direct experiments are that infrared radiation which was invisible to him and produced no effect on the human iris, nevertheless caused a strong con-

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striction of the owl's pupil. The indirect evidence is the ability of the tawny owl to capture living sparrows and mice in complete darkness, compared to its failure to secure these animals when they are dead. According to Vanderplank this speaks for the ability of the owl to see these animals by the far infrared emitted by them in their capacity as heat radiators. Moreover dead animals and horse meat which were not eaten in total darkness were easily eaten when illuminated by infrared radiation.

It is apparent that if the experiments of Vanderplank are as reported, the visual system in the nocturnal owl is of a completely different kind than heretofore recorded for vertebrates, and rather at variance with what may be expected in terms of the structure of the eye and of the retina. We therefore undertook to test this matter by studying the sensibility in the spectrum of a typically nocturnal owl (the long-eared owl, *Asio wilsonianus*) using as objective criterion the effect of light on the size of the pupil.

II

Sensibility to Infrared

The experiments reported by Vanderplank require the owl's eye to be sensitive (*a*) to the far infrared (near 9000 mμ) such as would be emitted by a black body radiator at a temperature of 40° C., and (*b*) to the near infrared such as is emitted by an ordinary tungsten incandescent lamp. Neither of these suppositions is correct, and the experiments of Vanderplank must have involved serious errors.

The sensibility of the tawny owl to the far infrared has been recently tested by Matthews and Matthews (1939). They found that the tawny owl's eye which gave retinal potentials when exposed to ordinary light, failed to show any potential when illuminated by the infrared radiation from a black body at temperatures between 40° and 400° C. Moreover, they demonstrated that the lens and vitreous humour of the eye are opaque to these radiations, as is to be expected from their high water content.

The long-eared owl, like the tawny owl, is an essentially nocturnal bird. We find that it is insensitive to the near infrared. This we established by exposing the dark-adapted owl to a ground glass screen which was illuminated by a 500 watt lamp through a Corning glass filter No. 255 transmitting no radiation below 750 mμ. When the lamp was about a meter from the screen, and so produced a large surface of intense infrared radiation, we were unable to observe any effect on the size of the pupil of the owl. If instead of the infrared filter we used the green Wratten filter No. 74, the result was a powerful contraction of the pupil. The precise apparatus will be described in the next section.

By means of a thermopile and galvanometer we measured the total energy transmitted (*a*) by Corning filter 255, and (*b*) by Wratten filter 74 with the infrared removed by a combination of copper sulfate solution and heat filter. The infrared transmitted by filter 255, which failed to produce any perceptible effect on the iris of the owl, contains about 5×10^6 times as much energy as the green light transmitted by filter 74 when it produced an easily perceptible contraction of the iris.

We repeated these experiments with the green and the infrared filters often enough and under a sufficient variety of conditions to be quite certain that the infrared produces no perceptible effect on the owl's pupil. We must conclude that Vanderplank's experiments involved some other factors than infrared radiation. Unfortunately Vanderplank gives no description of his apparatus, so that one cannot guess the origins of his aberrant results.

Having established the insensibility of the owl to infrared light, we felt that this datum would have more meaning if it were related to the sensibility of the owl's eye in the spectrum generally. In addition it seemed desirable to determine the effectiveness of the spectrum in producing a pupillomotor response, because there does not exist any quantitatively adequate description of the owl's sensibility in the spectrum which can be compared with modern determinations of the luminosity functions in the human eye (*cf.* Hess, 1912).

III

Apparatus and Method

The arrangement of the apparatus is illustrated in Fig. 1. The owl *A* sits on a rod in the wooden cage *C*, and is separated from the outside by a rectangular wire mesh *B* with openings 2 inches square. A large circular opening 2 feet in diameter in the front of the cage is covered with three layers of fine ground glass *G*. Three sheets of ground glass are used in order to provide a large, uniformly illuminated surface. Two ruby lamps *R* covered with paper are outside the mesh, and illuminate the owl so that its bright yellow iris is just adequately visible to the observer whose eye is at *O*. The owl is comparatively insensitive to red light, and the illumination by the two ruby lamps furnishes little visual stimulus; the resulting iris contraction is practically minimal. The pupil is large—nearly 1 cm. in diameter—and the iris may be seen as a fine bright band a fraction of a millimeter wide around the edge of the eye.

The illumination of the ground glass *G* is accomplished by the lamp *L* in the housing *T*. For experiments in the visible part of the spectrum, *L* is a 250 watt projection lamp while for work in the near infrared it is a similar 500 watt lamp. Various screens and filters are used to delimit the beam of light and to vary its intensity and color. *H* is a heat-absorbing filter, and *F* is any filter, neutral or colored, to control the color or intensity of the transmitted light. The whole assembly of lamp housing, screens, filters, and shutter *S* is on a moving table which can be placed at different distances from the ground glass screen.

The light intensity on the ground glass is varied by decimal neutral filters for discrete steps, and by changing the distance of the lamp from the screen to produce a continuous gradation between the decimal steps.

For work with the infrared, Corning filter No. 255 is used between the 500 watt lamp and the ground glass. The color temperature of this lamp is about 3,000°K; its energy maximum is therefore in the near infrared at about 1000 mμ. Since filter 255 transmits only above 750 mμ, most of the energy of the lamp passes the filter.

In the visible spectrum, different parts are isolated by Wratten monochromatic filters 70, 71A, 72, 73, 74, 75, and 76. Because these are gelatin filters, we use a Bausch and Lomb heat absorbing filter between them and the lamp. We calibrated the Wratten filters and the heat absorbing filter with Shlaer's photoelectric spectrophotometer (Shlaer, 1938). We also determined the energy distribution in the spectrum of the 250 watt lamp by measuring its color temperature in comparison with a lamp previously calibrated for different color temperatures. Using the transmission data for the filters and the energy distribution of the lamp, we computed the relative energy transmitted by each of the Wratten filters in the spectrum in conjunction with the heat filter. From this information we then determined the wavelength corresponding to the center of the energy transmitted by each filter combination. These are given later in Table I.

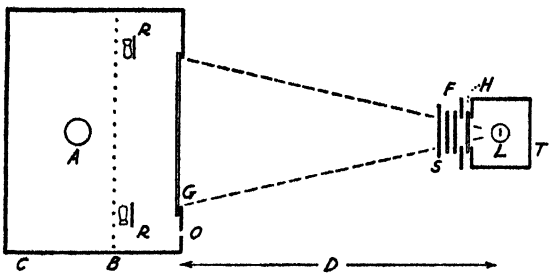

FIG. 1. Arrangement of apparatus. The owl at A sees the ground glass G which is illuminated by light from the lamp L after it has passed through various filters F for controlling its intensity and color. Further details are in the text.

It might seem that this indirect method of determining the relative energy transmitted by the various filters could be replaced by direct measurements with thermopile and galvanometer. Our experience, however, shows that it is difficult to eliminate completely the infrared transmitted by these filters; and since most of the lamp's energy is in the infrared, even the fraction which is transmitted by heat filters looms large in comparison with the energy in the particular part of the visible spectrum transmitted by a monochromatic filter.

The procedure in making the measurements is simple. First the owl is dark adapted for several hours. Then, the ruby lights are turned on, and the observer watches the iris of the eye. It is much better to focus attention on the bright though narrow iris band than on the much larger but darker pupil within it. When he feels certain of the size of the iris band, the observer signals for the opening of the shutter and the consequent illumination of the ground glass. The observer notes whether the iris band has changed in width as a result of the illumination. Depending on the response, the lamp is then brought nearer or farther, or a filter is put in or taken out. The observations are repeated in this way until an intensity is found which, when flashed suddenly on the ground glass screen, just produces a discernible increase in the width of the iris band—that is, a decrease in the size of the pupil.

We were pleasantly astonished at the precision which can be obtained by so simple a procedure, and therefore made no effort to measure the actual extent of the contraction of the iris in any objective way. The increase in width of the band which we could recognize with certainty is between 0.3 and 0.5 mm. and this minimal increase was adhered to throughout the experiments.

IV

Measurements

We made all our measurements with one owl kindly given us by Dr. G. K. Noble of the American Museum of Natural History. The seven Wratten filters were tested in no special sequence, and a series consisted in determining the relative energy required with each filter in order to produce the minimum observable pupil contraction. Over a period of several weeks we made five such series of measurements, in which we alternated as observer and manipulator.

TABLE I

Relative Energy for Minimal Iris Response in Different Parts of Spectrum ($E_{515\ m\mu} = 1$)

Wratten filter	76	75	74	73	72	71A	70
Central λ mμ	451	490	533	576	631	652	683
Relative energy	5.19	1.86	1.58	5.89	62.7	463.4	9863.0

The data are in Table I where each value is the average of the five separate determinations. For convenience the value of 1 is assigned to the energy at 515 mμ which turns out to be the position of minimum energy as determined graphically.

The measurements are plotted in Fig. 2 as circles connected with a continuous line. The ordinates are the negative logarithms of the relative energies in Table I. Logarithms are used in order to show the details more clearly in this wide range of energies;

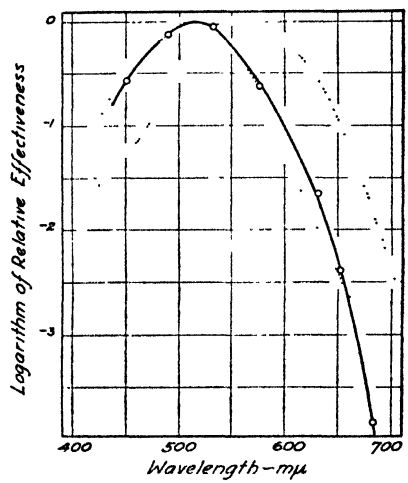

FIG. 2. Spectral sensibilities. The points connected with a continuous line are the average data of five series of measurements with the owl. The dotted lines represent the spectral sensibility curves of the human eye. The one at the left is at very low light intensities and represents rod function; the one at the right is at high intensities and represents cone function.

negative logarithms are for indicating the relative effectiveness of the spectrum.

It is apparent that the owl is very insensitive to red light. The energy in the red at 683 mμ necessary for a minimal iris response is approximately 10,000 times as great as that in the blue-green at 515 mμ. Extrapolation of the effectiveness curve in Fig. 2 toward the infrared makes it easily understandable why a relative energy content of 5,000,000 is not sufficient to produce even a minimal iris response. Thus, if one were to make a general statement about the owl's vision in the infrared, it would be the precise opposite of Vanderplank's, namely, that the owl is extremely insensitive to infrared radiation.

V

Spectral Luminosity Curves

The relative effectiveness curve in Fig. 2 is essentially a spectral luminosity curve (plotted logarithmically), since like the human spectral luminosity curves it records the reciprocal of the relative energy required in different parts of the spectrum to produce the *same physiological effect.*

It is to be regretted that this point is occasionally not understood, and measurements are published which cannot be compared quantitatively with standard data. Thus the practice of measuring the retinal potentials produced in an eye by a spectrum whose energy content is unknown (Piper, 1905) or whose energy content has been equalized (Granit and Munsterhjelm, 1937) yields data that cannot be compared with a visibility curve or even with an absorption spectrum curve of visual purple. For such comparisons (*cf.* Hecht, 1937) it is necessary to know the relative energy in different parts of the spectrum required to produce the *same physiological effect*, such as a constant motor response (Hecht, 1928) or a constant retinal potential (Graham and Riggs, 1935), or a constant number of optic nerve impulses (Graham and Hartline, 1935) or a constant amount of pupil contraction. It is easy to be misled by the equivocal definition of the human luminosity curve as the relative brightness of an equal energy spectrum; what this definition really means is the reciprocal of the energy in different parts of the spectrum required to produce a minimal or a constant visual brightness, not the relative brightness produced by a spectrum which is actually equal in energy throughout.

Since the data in Fig. 2 form a spectral luminosity curve, they may be compared directly with similar curves for the human eye. These are shown as a background in Fig. 2 in dotted lines. The one to the left is the luminos-

ity curve at low intensities and is from the data of Hecht and Williams (1922), confirmed by the later work of Sloan (1928) and of Weaver (1937) and represents the properties of rod vision. The one to the right is the luminosity curve at high intensities from the work of Gibson and Tyndall (1923) and represents practically pure cone vision. It is apparent that the measurements of the owl coincide with the dim luminosity curve of the human eye well within the range of individual variation. This confirms the rod nature of the owl's vision at low intensities and renders all the more secure the conclusion that it does not see in the dark by means of infrared radiation.

VI

Thresholds

There remains the problem which originally actuated Vanderplank, namely, how nocturnal owls manage to catch their prey in the (to us) extremely dim illuminations of the woods at night. In so far as this involves vision, it may be considered in terms of form discrimination and brightness.

We determined the brightness which produces the minimal effect on the iris of the owl. With the No. 74 (green) filter it is 1.5×10^{-4} millilamberts. The brightness was actually measured at a much higher value with a Macbeth illuminometer, and computed for the minimal value in terms of lamp distance and decimal filter transmissions. We also determined with the same green filter and the same apparatus the minimum brightness which we ourselves could see after complete dark adaptation. This is just about 4.0×10^{-7} millilamberts. Thus the light which produces a just perceptible iris contraction in the owl is nearly 400 times more intense than our absolute threshold.

The measurements of Reeves (1918) show that a minimal pupil contraction of 0.5 mm. in the human eye occurs at a light intensity which is about 1000 higher than the threshold. Such a change in pupil corresponds to the minimum observed in the owl's eye. Therefore, if the relation between pupil size and brightness in the owl is like that in our eye, the absolute threshold for the owl's eye is about 1/1000 of 1.5×10^{-4} millilamberts, or 1.5×10^{-7} millilamberts. However, one cannot put too much reliance on a computation of this kind, which may be off by a factor of 10.

A factor of 10 is much more important at low illuminations than at high, because visual acuity and form discrimination at low illuminations vary almost directly with intensity (*cf.* Hecht, 1937). Thus, all other things

being equal, if the owl's absolute threshold is one-tenth of ours, its visual acuity will be ten times ours at an illumination corresponding to our absolute threshold. In the illumination of the night woods this may give the owl enough form discrimination to account for its behavior.

SUMMARY

Infrared radiation (750–1500 mμ) produces no iris contraction in the typically nocturnal long-eared owl even when the energy content is millions of times greater than that of green light which easily elicits a pupil change. The energies in different parts of the visible spectrum required for a minimal iris response yield a spectral visibility curve for the owl which is the same as the human visibility curve at low light intensities. Functionally, the owl's vision thus corresponds to the predominantly rod structure of its retina, and the idea that nocturnal owls have a special type of vision sensitive to infrared radiation for seeing in the woods at night is erroneous.

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SOLUBILITY STUDIES ON PURIFIED TOBACCO MOSAIC VIRUS

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Solubility studies on many crystalline proteins have shown that even large molecules show solubility behavior in general accord with that expected for homogeneous substances from the phase rule. Such studies in the case of the crystalline hemoglobins and enzymes have yielded much valuable information as to their identity and purity (1, 2). Solubility theory should apply also to particles larger than the simple proteins and experiments with a bacteriophage by Northrop indicated that this material, although having a minimum molecular weight of about 500,000, possessed a fairly definite solubility (3). It has been shown that tobacco mosaic virus purified by centrifugation or by careful treatment with ammonium sulfate is homogeneous when examined either by sedimentation in the ultracentrifuge or by electrophoresis in the Tiselius apparatus (4, 5). These methods, however, show only that all of the material in the sample sediments in a centrifugal field or migrates in an electrical field at uniform rates. Although many other experiments also failed to provide evidence for inhomogeneity (6), it appeared desirable to study the solubility behavior of the virus. In the experiments to be described in this paper, solubility determinations were made on tobacco mosaic virus purified by different methods (7, 8), on samples isolated at varying intervals of time after inoculation (9), and on samples isolated from different host plants, in an effort to determine whether purified virus behaves as a homogeneous material from a solubility standpoint.

Materials and Methods

The virus samples were obtained by the more recent chemical procedures which involved only a few precipitations with ammonium sulfate and by ultracentrifugation with or without a preliminary precipitation with ammonium sulfate. When purified by the earlier procedures, the virus was recrystallized several times before it was used. When prepared by ultracentrifugation it was used without a preliminary crystallization other than that involved in the solubility determination. Samples prepared by ammonium sulfate precipitation were dialyzed on a rocking dialyzer (10) against distilled water until they failed to give a test for ammonia with Nessler's solution. The samples

prepared by ultracentrifugation were obtained by the sedimentation of the virus in the clarified juice or in concentrates prepared by precipitation of the virus with ammonium sulfate and re-solution in water. The virus pellets which separated during the ultracentrifugation were redissolved either in 0.1 M potassium phosphate buffer at pH 7 or in water and resedimented one or two additional times. The samples taken from plants 2 to 13 weeks after inoculation were portions of preparations isolated and described by Stanley (9) and used for sedimentation analyses by Wyckoff (5). Solutions in 0.1 M phosphate containing 2 to 3 per cent virus were stored at 4° for from 6 to 12 weeks before the solubility determinations were made.

Preliminary experiments showed that centrifugation in an angle head in an ordinary laboratory centrifuge served much better than filtration during a solubility determination to separate suspended crystals of virus from that in solution. Equilibrium was approached from the supersaturated side by adding to the sample of virus dissolved in 0.1 M phosphate buffer a weighed amount of ammonium sulfate sufficient to cause the crystallization of a part of the virus in solution. Equilibrium was approached from the undersaturated side either by crystallizing the virus at the isoelectric point with dilute phosphoric acid and extracting the sedimented virus, after the supernatant fluid had been thoroughly drained, with buffer containing ammonium sulfate (method 1); or by adding solid ammonium sulfate to the sample dissolved in a sufficiently small volume of buffer to cause the separation of all the virus, and then adding sufficient buffer to reduce the salt concentration to the desired level (method 2). In a few instances, solubilities were determined at room temperature. When this was done and the samples were centrifuged at 3000–4000 R.P.M. for 3 to 4 hours, the virus concentration in the supernatant liquid decreased only slightly after additional centrifugation. In most experiments, however, the virus suspensions, cooled to 0°, and stirred for 10 to 15 minutes, were allowed to stand for 24 hours at 4° and then were centrifuged at 3000–4000 R.P.M. for about 2 hours at the same temperature. The supernatant liquid was pipetted off and analyzed for protein nitrogen as previously described (7). The crystals which separated under these conditions, while very small in size, were the typical needle-like crystals. Because of the extremely small size of the crystals, it is difficult to be certain that amorphous material was absent in all cases. However microscopic examination did not show material of a definitely amorphous character.

RESULTS

Examples of the results obtained with virus samples purified by chemical procedures and by sedimentation in the ultracentrifuge before and after preliminary precipitation with ammonium sulfate, and the effect of storage in 0.1 M phosphate buffer or 20 per cent ammonium sulfate are shown in Table I. Samples purified by chemical procedures varied in solubility at room temperature in 5.25 per cent ammonium sulfate and 0.1 M phosphate at pH 5.6 from 0.26 to 1.5 mg. virus N per ml. Samples prepared by ultracentrifugation showed a similar variation. Some, at the concentrations tested, were entirely soluble in a concentration of ammonium sulfate at 4° which, for another sample, gave a solubility of about 0.3 mg. N per ml. at the same temperature. The solubility of a sample of virus varied greatly

with the concentration of ammonium sulfate used as solvent. A sample which was soluble to the extent of 0.93 mg. N per ml. in 11.18 per cent ammonium sulfate gave a solubility of 0.07 mg. N per ml. in 11.77 per cent ammonium sulfate. Storage for a month at 4° in 0.1 M phosphate buffer

TABLE I

Solubilities in Ammonium Sulfate and 0.1 M Potassium Phosphate Buffer of Tobacco Mosaic Virus Prepared by Different Methods

Experiment	Method of preparation	Mg. virus N per ml. suspension	Mg. virus N per ml. solution
1	Precipitation with ammonium sulfate (4). Recrystallized 5 times. Isolated 3 weeks after inoculation. Solubility at room temperature in 5.25 per cent ammonium sulfate at pH 5.6 from supersaturated side	1.58	0.26, 0.26
2	Prepared and solubility determined as in 1. Plants inoculated with virus obtained from a single lesion	1.58	0.36
3 *a*	Prepared and determined as in 1. Recrystallized several times	1.58	0.94
b	The same sample after standing 1 month at 4° in 0.1 M phosphate buffer at pH 7	1.58	0.21
4 *a*	Prepared and determined as in 1. Solubility determined without preliminary crystallization	1.58	1.50
b	The same sample after standing as in 3*b*		0.21
5	Prepared and determined as in 1. Solubility determined about a year after standing at 4° as a paste in 20 per cent ammonium sulfate	3.16	0.26
6 *a*	2 sedimentations in ultracentrifuge. Solubility at 4° in 11.18 per cent ammonium sulfate at pH 5.6 from supersaturated side	0.93	0.93
b	Same as 6*a*. Solubility in 11.77 per cent ammonium sulfate	0.93	0.07
7	Prepared and determined as in 6*a* in 11.77 per cent ammonium sulfate	0.93	0.05
8 *a*	3 sedimentations in ultracentrifuge. Solubility as in 6*a* at pH 6.4	0.76	0.25, 0.21
b	Same as 8*a*. From undersaturated side (method 2)	0.76	0.26, 0.26
c	Same as 8*b*	1.00	0.25, 0.26
d	Precipitated once with ammonium sulfate and purified by 3 sedimentations in ultracentrifuge. Solubility as in 8*b*	1.00	0.38, 0.35

or for about a year in 20 per cent ammonium sulfate reduced the solubility from relatively high values at room temperature to about 0.1–0.2 mg. N per ml. and repeated recrystallization at room temperature seemed to produce a similar result. Evidence has been obtained that these treatments cause an irreversible linear aggregation of the virus particles (11, 12). One precipitation with ammonium sulfate at 4°, which does not cause a measurable amount of such aggregation, also failed to cause any pronounced

change in the solubility of the virus. The specific activities of the original samples of virus used in the experiments given in Table I were not determined, but they were prepared by comparable procedures involving a minimum of treatment with ammonium sulfate or exposure to room temperature. In previous experiments it has been shown that such samples possessed comparable specific activities and gave sharp boundaries in the analytical ultracentrifuge.

TABLE II

Solubilities at 4° in 0.1 M Potassium Phosphate Buffer at pH 5.6 and 11.18 Per Cent Ammonium Sulfate of Tobacco Mosaic Virus Isolated from Plants Harvested from 2 to 13 Weeks after Inoculation

No.	Weeks after inoculation isolated	Method of preparation*	Mg. virus N per ml. solution†
1 *a*	2	Two sedimentations in ultracentrifuge. Solubility determined from supersaturated side	0.36
b		The same sample after standing 1 month longer at 4° in 0.1 M phosphate buffer at pH 7	0.54
2 *a*	3	Prepared and determined as in 1*a*	0.52
b		Precipitated once with ammonium sulfate and purified by 2 sedimentations in ultracentrifuge. Solubility as in 1*a*	0.14, 0.15
3 *a*	4	Prepared and determined as in 1*a*	0.19, 0.19
b		The same sample after standing as in 1*b*	0.08
4 *a*	5	Prepared and determined as in 1*a*	0.48
b		The same sample after standing as in 1*b*	0.17, 0.12
c		Prepared and determined as in 2*b*	0.02
5	6	Prepared and determined as in 1*a*	0.40, 0.62
6 *a*	7	Prepared and determined as in 1*a*	0.61, 0.48
b		The same sample after standing as in 1*b*	0.27
7 *a*	8	Prepared and determined as in 1*a*	0.50
b		The same sample. Solubility determined from undersaturated side (method 1)	0.48, 0.48
c		The same sample after standing as in 1*a*	0.40
d		Prepared and determined as in 2*b*	0.30
8	13	Prepared and determined as in 1*a*	0.77, 0.82

* The samples were stored in 0.1 M phosphate solution at 4° for from 6 to 12 weeks before the solubility determinations were made.

† Solubility determinations were made in all cases on 0.93 mg. virus N per ml. suspension.

The solubility experiments with the samples isolated by Stanley (9) from plants 2 to 13 weeks after inoculation were carried out from 6 to 12 weeks later. Those isolated from plants 2 to 8 weeks after inoculation gave relatively constant results (Table II). The mean value for six samples in ten determinations was 0.50 ± 0.02 mg. virus N per ml. at 4° in 11.18 per cent ammonium sulfate and 0.1 M phosphate at pH 5.6. The solubility of the 13 week sample, however, was about 0.8 mg. N per ml. under the same conditions. Virus that had been precipitated once in the cold with ammonium sulfate and then ultracentrifuged showed, in general, a decrease in solubility as compared with virus prepared from the same sap by ultra-

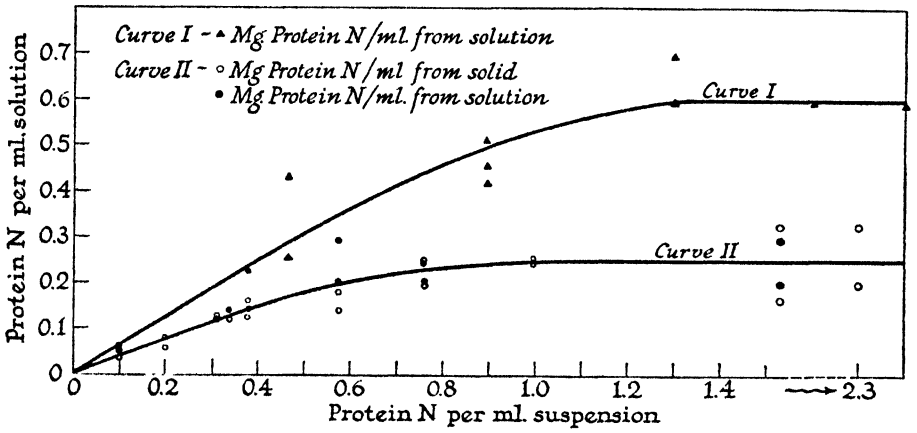

FIG. 1. Solubility of two ultracentrifuged preparations of tobacco mosaic virus in 0.1 M potassium phosphate and 11.18 per cent ammonium sulfate buffer: curve I at pH 5.6, curve II at pH 6.4.

centrifugation. A good deal of variation was found, however. Some samples were entirely insoluble after the ammonium sulfate treatment while others were nearly as soluble as the ultracentrifuged virus. The variation was probably due to differences in the length of time different samples had been allowed to stand in ammonium sulfate solution during the isolation procedure. Similarly, after standing for about a month longer in 0.1 M phosphate at 4°, the solubility of most of the samples tested decreased further.

The solubility data obtained with varying amounts of solid phase in the presence of a constant volume of solvent showed an increase in the amount of dissolved virus as the amount of solid phase was increased above that amount necessary to give a saturated solution. The results with two preparations obtained by ultracentrifugation are shown graphically in Fig. 1.

The data are not sufficiently precise to establish definitely the shape of the solubility curves. Relatively constant solubilities were obtained, however, as the amount of virus in suspension was increased beyond 1.3 mg. N per ml. in one case and beyond 1 mg. N per ml. in the other, but in both instances some solid phase separated in the presence of much smaller amounts of virus. In the case examined most completely (curve II) some solid virus separated or failed to dissolve even at one-tenth the concentration which seemed to give a constant solubility. Similar results were obtained with a sample purified by precipitation with ammonium sulfate. The solubility curves are not characteristic of a material consisting of only one solid phase but resemble those of solid solutions (13). A similar conclusion was reached when a crystallized sample of virus was extracted repeatedly with equal portions of the same solvent, for the amount of dissolved virus decreased progressively as the amount of virus used as saturating body decreased.

A possible explanation for the presence of solid phase at the lower virus concentrations was the formation of a fraction consisting of relatively insoluble and inactive virus as a result of the contact with the ammonium sulfate solution during the solubility determination. If this were the correct explanation then the specific activity of the crystallized virus would be expected to be lower than that of the virus in solution. To test this hypothesis the specific activities of the crystallized virus and the virus in solution from two solubility determinations (curve II, Fig. 1) in which the original virus concentrations were 0.38 and 0.57 mg. virus nitrogen per ml., respectively, were compared. The crystallized virus was dissolved in water in each case and these solutions and those containing the soluble virus were dialyzed until free of ammonium sulfate and analyzed for nitrogen. The dialyzed solutions of crystallized and soluble virus in one experiment were diluted to 10^{-5} gm. virus per ml. with 0.1 M phosphate buffer at pH 7 and compared on 42 half leaves of *Nicotiana glutinosa*. In two tests, 2392 and 2904 lesions were obtained for the solution of crystallized virus as compared with 2002 and 2602 lesions, respectively, for the virus in solution. In the second experiment, the dialyzed solutions were diluted to 2×10^{-6} gm. virus per ml. and compared on 37 half leaves of *Phaseolus vulgaris* L. var. Early Golden Cluster. In two tests, 253 and 627 lesions were obtained for the crystallized virus as compared with 388 and 508, respectively, for the virus in solution. When examined statistically on the basis of half-leaf units by "Student's" method (14), the crystallized virus in two tests was significantly more active than that in solution, the virus in solution was significantly more active in one test and in the other

the difference in lesion counts was not significant. When the total numbers of lesions obtained by each treatment were combined and evaluated similarly, the crystallized virus rather than that in solution proved significantly more active. It may be concluded, therefore, that the fraction of virus which precipitated did not consist of inactive or largely inactive virus.

The question of a change in tobacco mosaic virus as a result of treatment with ammonium sulfate was also studied by comparing the activities of samples of virus isolated from portions of the same infected sap by ultracentrifugation and by ammonium sulfate precipitation in the cold. In eight tests in which a total of 305 half leaves of *Phaseolus vulgaris* was used for the comparison of samples isolated from time to time, a total of

TABLE III

Solubility Experiments with Tobacco Mosaic Virus from Tobacco and Tomato Plants

	Mg. virus per ml. supernatant after centrifugation for 2 hrs. at about 4°C.
(1) 50 mg. virus from tobacco plants in 10 cc. 0.1 M phosphate at pH 7 crystallized with 975 mg. $(NH_4)_2SO_4$	4.39
(2) 50 mg. virus from tomato plants in 10 cc. 0.1 M phosphate at pH 7 crystallized as in (1)	4.49
(3) 50 mg. crystals of virus from tobacco plants extracted with 10 cc. 0.1 M phosphate at pH 7 + 975 mg. $(NH_4)_2SO_4$	4.04
(4) 50 mg. crystals of virus from tomato plants extracted as in (3)	4.15
(5) 5 cc. of supernatant from (3) used to extract crystals from (4)	4.11
(6) 5 cc. of supernatant from (4) used to extract crystals from (3)	4.09

11,160 lesions was obtained for the samples prepared by ultracentrifugation as compared with 11,202 for the samples prepared by precipitation with ammonium sulfate. If the treatment with ammonium sulfate resulted in an irreversible change in virus in these experiments, it was not accompanied by a significant loss of virus activity. It should be noted, however, that a decrease in specific activity or a loss of active virus corresponding to less than about 10 per cent of the original might not be detected by the infectivity tests.

Solubility of Virus from Different Host Plants.—Preliminary experiments in which the solubilities of tobacco mosaic virus isolated from Turkish tobacco and tomato plants by chemical methods were compared, showed that about the same solubility behavior was obtained for virus isolated from either host provided the samples were isolated by the same procedures from plants inoculated for the same periods of time (7). Similar experi-

ments were carried out on ultracentrifuged preparations of virus from tobacco and tomato plants. The juice was obtained from young plants about a month after inoculation and the virus was precipitated once in the cold with 20 per cent ammonium sulfate. It was redissolved in 0.1 M phosphate buffer and sedimented twice in the ultracentrifuge. The experiments on two samples obtained in this manner are summarized in Table III. It may be seen that under the conditions used, about the same solubility was obtained for the tomato as for the tobacco sample when the solubility was approached either from the supersaturated or the undersaturated side. When the saturated solution obtained from the tobacco sample was used to extract the crystals which separated from the tomato sample, a slight decrease rather than increase in the amount of dissolved virus took place, and a similar result was obtained when the saturated solution from the tomato sample was used to extract the crystals which separated from the sample isolated from tobacco plants.

DISCUSSION

The wide variation found for the solubility of different samples of purified tobacco mosaic virus prepared by precipitation with ammonium sulfate and by ultracentrifugation, and the type of solubility curve obtained in the presence of varying amounts of solid phase indicate, contrary to the results of ultracentrifugal and electrophoretic analyses, that the purified virus is not a homogeneous material. The results are not readily explained either by the presence of inactive impurities, by the reversible or irreversible formation of inactive virus, or by failure to attain equilibrium during the solubility determinations. They may be explained by variations produced in the virus itself either during growth under different conditions or during isolation. Evidence for a small amount of variation in tobacco mosaic virus has been obtained previously (15) by the isolation of mutant strains from plants infected with a single infectious unit of the ordinary strain. These were recognized because of characteristic signs of disease on infected plants different from those of ordinary tobacco mosaic and have been carried serially through many generations of plants. The number and amount of such strains which are formed during the production of the virus in the host plants are not believed to be sufficient to account for the type of solubility curve found. In view of the fact that the more soluble and the less soluble fractions are equally infectious and, in the limited number of tests made, produce typical tobacco mosaic disease, it seems more likely that the solubility of the infectious agent itself may vary. It is possible that the less soluble virus may be derived from the

more soluble by a process similar to that taking place when the virus ages *in vitro*, or it may be that during the multiplication of the original infective unit other units differing slightly in solubility but causing typical tobacco mosaic are produced. The solubility data provide evidence that the purified virus is better described, as has already been indicated (16), as a family of closely related substances rather than as a single chemical individual. The experiments with the virus from tobacco and tomato plants show, however, that remarkably comparable virus may be obtained from plants of different genera when the latter are grown under the same conditions and the virus is isolated by similar procedures.

SUMMARY

Different samples of purified tobacco mosaic virus show a relatively wide variation in solubility in ammonium sulfate solution. This variation and the type of solubility curve obtained in the presence of varying amounts of solid phase show that the purified virus whether isolated by mild treatment with ammonium sulfate or by ultracentrifugation is not a homogeneous chemical substance but contains more soluble and less soluble virus fractions of comparable specific activities. Long contact with strong ammonium sulfate solutions or 0.1 M phosphate buffer results in a decrease in solubility. The variation in the solubility of samples isolated from different plants by the same method seems to depend in part on the length of time the plants are inoculated before they are cut, and probably also on the conditions under which they are grown. Virus preparations isolated from plants of different genera grown under the same conditions and inoculated at the same time, however, behaved like identical substances in solubility experiments.

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SUMMATION OF ELECTRICAL POTENTIAL OF LIVING MEMBRANES (FROG SKIN): A MODEL OF THE ELECTRICAL ORGAN OF FISHES

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INTRODUCTION

For the interpretation of the electrical field of the organism as a whole (Burr and Northrop, 1939) the possibility of algebraical summation of membrane potentials throughout the body must be considered. Frog skin is particularly suitable for the construction of models of summated potentials, as it can be removed without injury and survives several hours in Ringer's solution. Moreover, this tissue performs many of the functions of the plasma membrane of single cells such as respiration, water intake, and ionic exchange (Dean and Gatty, 1937; Krogh, 1937), and is therefore of interest in connection with the problem of the summation of cellular E. M. F.'s (Rosene, 1935). The most striking example of the possible summation of the potential of single units is the electric organ of certain fishes. Thus the 400 elements in series of the electric ray, producing a total of 30 volts, must develop an average of 75 mv. apiece (Hill, 1932, p. 12), which is a typical value for frog skin. The following experiments were designed to demonstrate the summation of the potentials of pieces of skin arranged in series in tubes of Ringer's solution.

Methods

Pieces of frog skin from the ventral and dorsal region of the trunk were tied on the blunt end of medicine droppers (external diameter 9 mm.) mounted on No. 4 rubber stoppers inserted in 11 cm. lengths of glass tubing (diameter 21 mm.) as indicated in Fig. 1. Each tube was filled with Ringer's solution through a side arm and the units linked by short lengths of rubber tubing filled with Ringer's solution fitted on the projecting ends of the medicine droppers. All the skins faced the same direction; *i.e.*, in each tube one skin was tied with the negatively charged outside outward and the other skin in the opposite direction (indicated by "normal" and "reversed" polarity in Tables I and II). The terminal tubes contained only one skin each and into these dipped Ag-AgCl electrodes leading to a type K potentiometer or a Micromax recording potentiometer. The summation potentials, however, were beyond the upper limit (100 mv.) of the recorder. The potential of each skin was measured separately before connecting

the holders in series. The potential of the entire chain was taken and also the resistance (60 cycle conductivity bridge) and amperage. The potential of each skin was measured again when the model was dismantled. The pH of the Ringer's solution at the end of each experiment as determined by a glass electrode was 7.4–7.6, which is in the optimal range for frog skin potential (Barnes, 1939). The Ringer's was thoroughly oxygenated at the beginning of each experiment. Owing to the length of the model a thermostat was not used. The temperature was taken at every reading but was not a disturbing factor. Owing to the variation in potential of different frogs the sex and weight of each animal are recorded in Tables I and II. The time elapsing between the initial and final reading of the potential of each skin is recorded in the column marked "Age" in Tables I and II.

RESULTS

As will be seen in Table III the potentials built up in the eight models approached the value predicted from the potentials of each skin measured

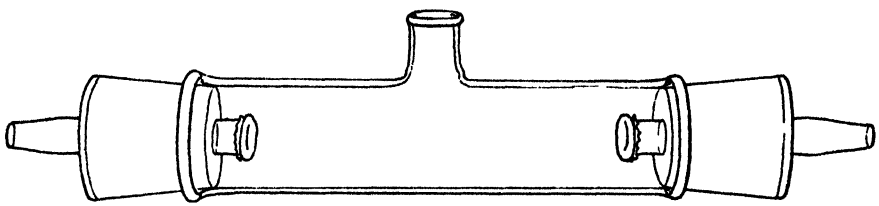

FIG. 1. Tube for holding frog skins in model for summation of potential (see text).

separately. The discrepancies may be ascribed to three factors: (*a*) the natural fluctuations which occur in all normal frog skin preparations. Thus in a model of twenty skins, an hour may elapse between the potential reading of the first skin and that of the completed chain. (*b*) The determination of the resistance alters the potential. (*c*) Changes in the tension on the skin occurring during the assembling of the model modify the potential (Barnes and Coe, 1940).

The data on amperage and resistance of the models are presented in Table IV. The needle of the ammeter remained deflected for many hours indicating a continuous output of electrical energy (Francis, 1933), which is also suggested by the temperature characteristics of 10,000 and 17,000 calories previously determined (Barnes, 1940).

SUMMARY

Frog skins arranged "in series" in tubes of Ringer's solution exhibit summation of electrical potential which helps to explain the nature of electrical fields in the organism as a whole and in the electrical organ of fishes.

TABLE I

Summation of Skin Potentials. Model No. 1

All skins from same frog, male 33 gm., 2 April, 22.7–25°C. Skin No. 1 next to positive electrode.

Skin No.	Origin	Polarity	Initial E. M. F.	Final E. M. F.	Age
			mv.	*mv.*	
1	Anterior ventral	Reversed	29.2	37.2	1 hr., 11 min.
2	Posterior ventral	Normal	41.2	32.6	1 hr., 11 min.
3	Posterior dorsal	Reversed	18.4	4.0	1 hr., 10 min.
4	Anterior ventral	Normal	10.0	8 6	1 hr., 14 min.
			98.8	82.4	
			(Calculated)	(Calculated)	
			105.0	87.0	
			(Measured)	(Measured)	

TABLE II

Summation of Skin Potentials. Model No. 4

Skins from seven frogs. 5 April, 20–22.8°C. Skin No. 1 next to negative electrode.

Skin No.	Frog No.	Sex and weight gm.	Origin	Polarity	Initial E. M. F.	Final E. M. F.	Age
					mv.	*mv.*	*min.*
1	1	♀ 36.0	Anterior ventral	Normal	33.0	26.5	64
2	1	♀ 36.0	Posterior ventral	Reversed	92.5	64.5	60
3	2	♀ 33.5	Anterior ventral	Normal	32.8	18.8	62
4	2	♀ 33.5	Posterior ventral	Reversed	91.8	70.0	59
5	3	♂ 33.5	Anterior ventral	Normal	33.8	20.6	59
6	3	♂ 33.5	Posterior ventral	Reversed	95.3	48.4	57
7	4	♀ 41	Anterior ventral	Normal	41.3	23.6	57
8	4	♀ 41	Posterior ventral	Reversed	67.6	24.5	54
9	5	♂ 27	Anterior ventral	Normal	23.0	10.0	56
10	5	♂ 27	Posterior ventral	Reversed	70.0	48.1	55
11	6	♂ 29.5	Anterior ventral	Normal	42.7	31.3	55
12	6	♂ 29.5	Posterior ventral	Reversed	92.1	62.6	55
13	7	♀ 44.5	Anterior ventral	Normal	32.9	31.8	54
14	7	♀ 44.5	Posterior ventral	Reversed	62.5	78 7	53
					821.3	568.4	
					(Calculated)	(Calculated)	
					758.6	597.1	
					(Measured)	(Measured)	

TABLE III

Summation of Skin Potentials. Data from Eight Models

Model No.	No. of skins	Initial E. M. F.		Final E. M. F.	
		Calculated	Measured	Calculated	Measured
		mv.	*mv.*	*mv.*	*mv.*
1	4	98.8	105.0	82.4	87.0
2	12	316.6	319.5	292.5	342.5
3	12	280.5	223.6	210.0	192.2
4	14	821.3	758.6	549.1	597.0
5	18	—	—	258.9	398.3
6	18	534.6	581.7	387.0	436.0
7	18	350.2	300.0	345.3	430.0
8	20	842.2	650.0	61.0	51.3

TABLE IV

Potential, Amperage, and Resistance of Frog Skins

Model No.	Potential before resistance reading	Amperage before resistance reading	Resistance of entire circuit
	mv.	*microamperes*	*ohms*
2	235.8	5.0	36,300
3	223.0	6.0	39,500
4	747.1	12.0	51,300
	510	5.0	64,500
	(9 hrs. later)	(9 hrs. later)	(9 hrs. later)
6	562.0	8.0	67,000
	447.6	5.0	76,000
	(12 hrs. later)	(12 hrs. later)	(12 hrs. later)
7	300.0	4.0	63,000
	350.0	4.0	75,000
	(10 hrs. later)	(10 hrs. later)	(10 hrs. later)
8	650.0	6.5	95,000
	147.0	3.0	78,000
	(21 hrs. later)	(21 hrs. later)	(21 hrs. later)

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ELECTROPHORETIC HOMOGENEITY OF PREGNANT MARE SERUM GONADOTROPHIN*

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Impurities in physiologically active substances may lead to erroneous concepts of their action. Such a situation is actual when the substance in question is protein in nature. The development by Tiselius (1) of the technique of electrophoresis has made this a valuable tool in characterizing proteins. We (2) have already shown that a highly purified and potent preparation of the pituitary lactogenic hormone behaves like a *single* substance in the Tiselius apparatus. The present study is a report to the effect that the same technical methods show electrophoretic homogeneity in a preparation of the gonadotrophic hormone in pregnant mare serum (PMS)—a protein substance (3).

EXPERIMENTAL

Gonadotrophic Preparation.[1]—The method of preparation was essentially that described by Goss and Cole (4) and we are indebted to them for making their findings known to us in advance of publication.

Forty liters of pregnant mare serum were brought to pH 8.98 and acetone equal to 90 per cent of its total volume was added. The precipitate was removed and washed with 40 liters of 50 per cent acetone. The residue was filtered off and discarded. The supernatants were combined and acetone was added to a specific gravity of 0.937. Acid was added to pH 6.0 and the precipitate which formed on standing was filtered off and discarded. Acetone was added to the filtrate to a specific gravity of 0.880; the dried precipitate weighed 19.779 gm. and exhibited a potency of 100 RU[2] per mg. This crude powder was extracted with 40 per cent acetone of pH 6.40 solution. The

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[1] Commercially known as gonadin.

[2] One rat unit (RU) is defined as the smallest amount of a gonadotrophin which on intraperitoneal injection in normal immature female rats (3 days injection) causes development of follicles and estrous uterus in at least 2 out of 3 rats.

insoluble material was discarded. The supernatant was brought to 50 per cent acetone and pH 5.50. The precipitate was removed and dried. The pH was then adjusted to 4.5 and the precipitate was removed and dried. The powders from the pH 5.5 and pH 4.5 precipitations were combined and treated as above except that the original extraction was made with 50 per cent acetone instead of 40 per cent acetone. The final 50 per cent acetone (pH 4.5) precipitate weighed 667 mg. and possessed a biological activity of 1000 RU per mg.

Electrophoresis Experiments.—The Hellige apparatus developed by Tiselius (1) was employed; the technique has been described previously (2). The protein solution which had been dialyzed and possessed the same pH and conductance as the buffer solution was then subjected to electrophoresis. The homogeneity and the migration of the boundary was determined by the schlieren method. The hydrogen ion concentration of the solution was measured with the glass electrode and its conductance with the usual Wheatstone bridge type of circuit and a Washburn conductivity cell. All experiments were performed at 1.5°C.

Ketene Experiments.—The ketene was generated by an apparatus designed by one of us (5). The protein to be acetylated was dissolved in pH 5.7 M acetate buffer. The solution contained 5 mg. of protein per cc. The experiments were carried out at room temperature.

Carbohydrate Determination.—The sugar content of the preparation was estimated by the method of Sorensen and Haugaard (6). Into 15 cc. of 60 per cent H_2SO_4 were pipetted 1 cc. protein solution and 2 cc. 2 per cent orcinol in 60 per cent sulfuric acid, a blank omitting the orcinol being set up for each solution. After thorough mixing the solutions were heated in a water bath at 80 ± 2°C. for 20 minutes, plunged into ice water, and cooled in the dark. The color developed was measured in a Cenco-Sanford-Sheard photelometer using a blue filter. The sugar content of the solution was read off from a calibration curve which was made using galactose as the reference carbohydrate.

Protein Nitrogen and Amino Nitrogen Determinations.—Estimation of protein nitrogen was carried out in triplicate by the micro Kjeldahl method using selenium oxychloride as catalyst for digestion. Amino nitrogen determination was performed using the Van Slyke gasometric apparatus. The nitrous acid was allowed to react for 6 minutes at room temperature.

Tyrosine and Tryptophane Determinations.—Lugg's (7) modified method of Folin and Ciocalteu was used and 30 mg. of the preparation were dissolved with 0.5 cc. of 5 M NaOH in a small sealed test tube. The test tube was then put into a steam bath for about 35 hours. The tyrosine and tryptophane in the hydrolyzed solution were determined in the same manner as described by Lugg except the colorimetric comparisons which were made in a Cenco-Sanford-Sheard photelometer instead of a colorimeter.

RESULTS

Electrophoretic Homogeneity and Isoelectric Point of the Preparation.—Fig. 1 shows the migration of the boundary resulting from the electrophoresis of 1 per cent solution, taken at 20 minute intervals in an electrical field having a potential gradient of 8.08 volts per cm. The schlieren band of the ascending boundary in another experiment using 0.8 per cent solution

is shown in Fig. 2. The average mobility[3] of the protein was then found to be 10.51×10^{-5} cm.^{2}/volt/sec. in phosphate buffer of pH 7.53 and ionic strength 0.05 at 1.5°C. The well defined and sharp boundary observed in these experiments indicates a high degree of purity of the substance. It must be admitted that all that is established is that possible contaminants

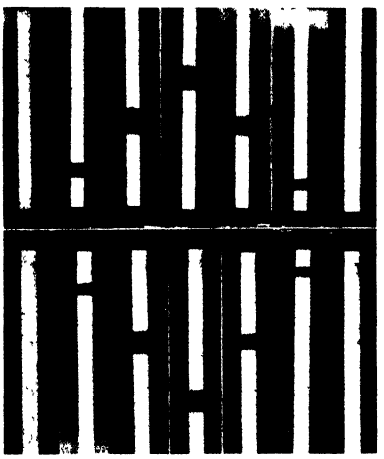

FIG. 1

FIG. 2

FIG. 1. Schlieren bands of PMS gonadotrophin. Exposures were made at 20 minute intervals and the current was reversed after the fourth exposure.

FIG. 2. Schlieren bands of the ascending boundary. Exposures were made at 16 minute intervals.

have the same or nearly the same mobility as the main component of our preparation so that resolution into two boundaries was not permitted.

[3] It will be seen in Table I that the mobility of a more dilute protein solution (*ca.* 0.2 per cent) is also 10.38×10^{-5} in the same buffer. Thus, the protein concentration does not change the electrophoretic mobility. However, Davis and Cohn (8) observed that the mobility of hemoglobin is directly proportional to the concentration. Recently, Stenhagen and Teorell (9) arrived at an opposite conclusion from their studies on thymonucleic acid.

We also found that the mobility varied with the potential gradient.

pH	E	$u \times 10^{-5}$	$(\Delta u/\Delta E) \times 10^{-9}$
4.01	7.76	4.22	0.051
4.01	15.52	4.62	
4.55	7.73	5.68	0.030
4.55	16.80	5.95	

The change of mobility per unit gradient increase was about one-tenth of that obtained by Stenhagen and Teorell. This phenomena may be explained by the Wien effect (10) of electrolyte solution.

We have also determined the mobility of the hormone in a wide range of hydrogen ion concentrations. The mobility studies were made with about

TABLE I

Electrophoretic Mobilities of PMS Gonadotrophin at 1.5°C. in Buffer Solutions of Varying pH and Constant Ionic Strength (0.05)

Experiment No.	Buffer	pH	$u \times 10^{-5}$
L 594	Potassium chloride	1.64	+3.67
L 584	Phthalate	2.32	+1.22
L 582	Phthalate	3.02	−1.51
L 590	Phthalate	3.41	−3.34
L 591	Acetate	4.01	−4.22
L 587	Acetate	4.55	−5.68
L 578	Acetate	5.40	−7.42
L 608	Phosphate	7.53	−10.38

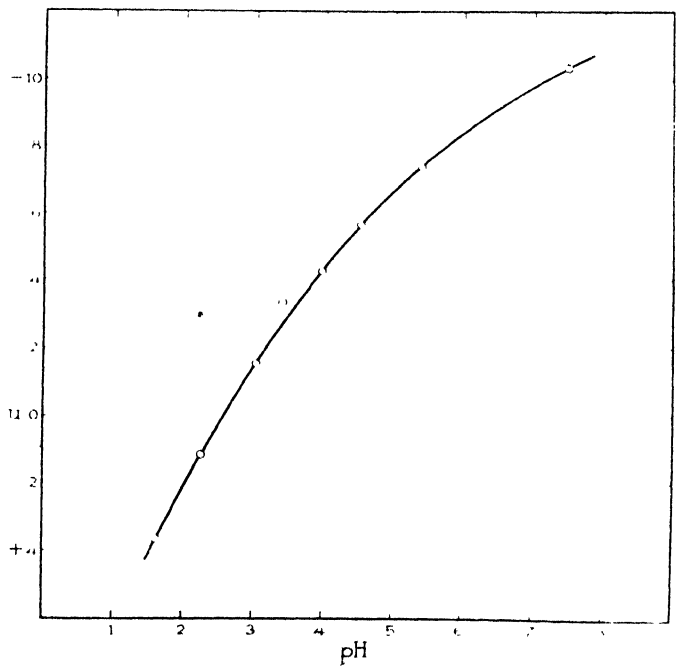

FIG. 3. Mobility of PMS gonadotrophin at different hydrogen ion concentrations (temperature 1.5°C.).

0.2 per cent solution in buffers of ionic strength 0.05 and constant potential gradient (*ca.* 8.0 volts per cm.). The results are given in Table I and Fig. 3. The last column of Table I is the mobility of the protein in cm.²/sec./

volt and the plus and minus signs refer to the charge in the protein. The isoelectric point[4] of the hormone was found at pH 2.60–2.65 and the value of $\frac{du}{d\mathrm{pH}_0} = 4.0 \times 10^{-5}$. It is not surprising that the isoelectric point lies in this high acid region, because most of the mucoproteins (11) possess this characteristic property. It is of interest to compare these results with those obtained for the gonadotrophic hormone in the urine of pregnant women (prolan). Recently Gurin, Bachman, and Wilson (12) have found the isoelectric point of their highly purified prolan at pH 3.3–3.4. The different isoelectric points of the two hormones suggest that there is a different ratio of acid groups to basic groups in the two molecules and the differing ratios may be related to the differences in the biological action of the two hormones.

Stability.—The preparation originally contained about 1000 RU per mg. When the dry powder[5] stood at room temperature in the desiccator for about 2 weeks, the potency decreased to one-half and it became stable at this potency. The electrophoretic homogeneity, however, remained the same, while the solution which had been used in eight electrophoresis experiments during a period of 35 days at 1.5°C. showed no loss of potency.

The hormone was stable in acid solution. When 3 mg. of the protein were dissolved in 10 cc. of 0.5 M HCl and kept at room temperature for 4 hours, the biological potency was not changed. On the other hand, the hormone was greatly deactivated in NaOH solution under similar conditions. It was found that the potency became less than 20 RU per mg.

Some Chemical Constituents of the Hormone.—Some chemical constituents of the hormone are summarized in Table II. Of interest is the carbohydrate content[6] which obviously classifies the hormone as a glycoprotein; whether it is essential for its biological activity is not yet clear.

It has been pointed out by Cohn (13) that tryptophane and tyrosine are

[4] It is of interest to note that Goss and Cole (4) found that the isoelectric point of their preparation in 50 per cent acetone lies between pH 5.5 and pH 4.5. The shift of isoelectric point toward the alkaline side indicates that the dissociation constant of the acid groups in 50 per cent acetone is much smaller than that in aqueous solution while the dissociation constant of basic groups changes to a smaller extent.

[5] An independent observation on the instability of the dry powder was made by Dr. H. H. Cole (personal communication).

[6] The carbohydrate content of a crude PMS preparation was also investigated. The potency of the crude PMS was about 50 RU per mg. and it contained only 6.0 per cent carbohydrate. Thus the carbohydrate determination may be taken as a measure of PMS potency in the purification of the hormone. The carbohydrate in the hormone was identified by orcinol method to be galactose, using blue and green filters in the Cenco photelometer.

particularly useful in the estimation of the minimal molecular weights of the proteins. Thus we calculate the minimal molecular weight of the hormone based on the tryptophane content to be 15,000. If we assume that there are two molecules of tryptophane and six molecules of tyrosine in the protein, the molecular weight will be about 30,000 gm.

Action of Ketene.—The effect of ketene on a crude preparation of PMS has been reported recently by us (14). The same results were obtained by applying the same technique to this highly purified and potent sample. Ketene treatment of the protein for 30 minutes caused a marked loss of physiological activity; the treated substance has a potency of less than 1 RU per mg. Since the amino groups in sugar-rich proteins are completely acetylated (14) by ketene at room temperature within 30 minutes, the results suggest that the physiological activity of PMS gonadotrophin

TABLE II

Some Chemical Constituents of the Gonadotrophin in Pregnant Mare Serum

Composition	Percentage
Protein nitrogen	10.60
Amino nitrogen	0.46
Carbohydrate	14.10
Tyrosine	3.54
Tryptophane	1.37

depends on the free amino groups. This finding is in accordance with the observations of Cartland and Nelson (3). They found that 84 per cent of the hormone was destroyed by exposing the hormone to 4 per cent formaldehyde for 3 hours at pH 8.0. It is known that formaldehyde reacts only with the free amino groups under the cited conditions.

It may be noted in this connection, that Bischoff (15) could not detect any significant difference between the gonadotrophin in pregnant women's urine and that in pregnant mare serum. In our present and previous studies we (14) have shown that the free amino groups are important for the biological action of PMS gonadotrophin while blockage of the amino groups by acetyl radicals does not essentially disturb the physiological activity of human chorionic gonadotrophin.

A similar conclusion as to differences in the chemical behavior of gonadotrophins has been reached by studying the specific action of nitrous acid (16). The PMS gonadotrophin is inactivated quickly (one-half hour) by nitrous acid at 0°C. whereas human chorionic gonadotrophin is inactivated *very slowly.*

SUMMARY

A highly purified and potent gonadotrophin in pregnant mare serum has been prepared. The preparation has been shown to be electrophoretically homogeneous in the Tiselius apparatus. The mobilities of the substance have been determined over a wide range of hydrogen ion concentrations. The isoelectric point lies at pH 2.60–2.65 and the value of $\frac{du}{d\mathrm{pH}_0}$ is 4.0×10^{-5}.

Some chemical constituents have been studied. From the tryptophane and tyrosine content the molecular weight of the hormone is estimated to be 30,000.

The hormone has been subjected to acetylation by ketene in aqueous solution at room temperature and the result suggests again the essentiality of free amino groups for the biological activity of the hormone. In this respect it is to be contrasted with human chorionic gonadotrophin.

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SEPARATION OF POTASSIUM ISOTOPES IN VALONIA AND NITELLA

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(Received for publication, April 15, 1940)

A. K. Brewer[1] reports that in certain cases the ratio $K^{39} \div K^{41}$ in the organism is not the same as in the environment. This situation appears to exist in *Valonia*[2] and *Nitella*[3] according to determinations made by Dr. Brewer to whom the author has sent samples of sap. The author desires to make grateful acknowledgment of Dr. Brewer's kindness.

The cells of *Valonia* were removed from the sea water, rinsed in distilled water, and pierced by a glass tube tapered to a fine capillary. By means of gentle pressure or suction the tube was filled with sap which was transferred to quartz or Pyrex bottles.

The *Nitella* cells were rinsed with distilled water and cut open to allow the sap to flow out into quartz or Pyrex vessels.

The results are shown in Table I. In the opinion of Dr. Brewer, they indicate some separation of K^{39} from K^{41} by *Valonia* and *Nitella*.

It is evident that this separation cannot be due to diffusion. The diffusion path in both cases is very short.

It seems possible that the separation depends on a higher partition coefficient, S, for K^{41} than for K^{39} (S = concentration in the non-aqueous protoplasmic surface layer $\div$ concentration in the external solution). A similar explanation has been suggested[4] for the fact that K^+ is taken up by *Valonia* in preference to Na^+ and this can be imitated to a certain extent by guaiacol which takes up more K^+ than Na^+. This is in accordance with the rule of Shedlovsky and Uhlig[5] which states that the partition coefficient increases with the ionic radius. On this basis we might expect K^{41} to be taken up more than K^{39}.

* Work completed by Dr. Jacques before his death in February, 1939.

[1] Brewer, A. K., *J. Am. Chem. Soc.*, 1936, **58**, 365, 370; *Ind. and Eng. Chem.*, 1938, **30**, 893. Lasnitzki, A., and Brewer, A. K., *Nature*, 1938, **142**, 538.

[2] *Valonia macrophysa*, Kütz., collected in Bermuda.

[3] *Nitella flexilis*, Ag., collected in the vicinity of New York City.

[4] *Cf.* Osterhout, W. J. V., *Bot. Rev.*, 1936, **2**, 283.

[5] Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933–34, **17**, 549, 563.

In view of this the following experiment was made. A solution of 0.2 M KOH was shaken with a mixture of 70 per cent guaiacol + 30 per cent *p*-cresol: this will be called G.C. mixture for convenience. The K^+ was extracted from the G.C. mixture by shaking with 0.5 M H_2SO_4 and was reconverted to KOH: this was shaken with fresh G.C. mixture; from this the K^+ was again removed by H_2SO_4, converted to KOH, and again shaken with G.C. mixture, and so on. After 13 repetitions[6] the ratio of $K^{39} \div K^{41}$ in the G.C. mixture was 14.10 ± 0.04. At the start it was 14.20 ± 0.04. Dr. Brewer regards this change as possibly significant.

TABLE I

Solution		$K^{39} \div K^{41}$
Bermuda sea water		14.20 ± 0.03
Valonia sap	Sample I	13.85 ± 0.05
	Sample II	13.85 ± 0.05
Nitella sap	Sample I	13.85 ± 0.03
	Sample II	14.00 ± 0.03
KOH before shaking		14.20 ± 0.02
K taken up by guaiacol mixture	Sample I	14.10 ± 0.04
	Sample II	14.09 ± 0.03

This result suggests that further experiments with organic substances may prove interesting.

SUMMARY

The ratio of $K^{39} \div K^{41}$ appears to be lower in the sap of *Valonia* and *Nitella* than in the environment, indicating that the living cell can separate these isotopes to some extent.

Experiments with a mixture of guaiacol and *p*-cresol suggest that a similar separation may occur here but further experiments are needed.

[6] A similar result (decrease of the ratio $K^{39} \div K^{41}$ in the G.C. mixture) would presumably be achieved by shaking the G.C. mixture once with a sufficiently large volume of aqueous KOH.

ACTION CURVES WITH SINGLE PEAKS IN NITELLA IN RELATION TO THE MOVEMENT OF POTASSIUM

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Although closely related, *Chara*[1] and *Nitella*[2] show striking differences. The action curve in *Nitella* has two peaks but in *Chara* there is only one. The outer protoplasmic surface in *Nitella* is sensitive to K^+ but this is not true of *Chara*.

It is of decided interest to find that these differences can be abolished by appropriate treatment. When cells of *Nitella* are leached in distilled water the outer surface becomes insensitive[3] to K^+ and at the same time the form of the action curve approaches that of *Chara* and shows only a single peak. The treatment with distilled water[3] commonly removes the irritability[4] as well as the sensitivity[5] to K^+ but cells are occasionally met with in which the irritability persists after the sensitivity to K^+ disappears. These present some interesting features.

Under normal conditions, cells of *Nitella* have an outwardly directed (positive[6]) P.D. of about 100 mv. due chiefly to the outwardly directed concentration gradient[7] of K^+ across the inner protoplasmic surface *Y*.

When an action current appears this P.D. disappears, partially or completely, producing the first movement of the action curve, *i.e.* the spike, or *o* movement, as seen in Fig. 1.

This is presumably due to an increase in the permeability of *Y* which

[1] *Chara coronata*, Ziz. The large cells, resembling those of *Nitella*, are not covered with a layer of small cells as in most species of *Chara*.

[2] *Nitella flexilis*, Ag.

[3] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 105.

[4] By this is meant the ability to give propagated action currents on electrical stimulation.

[5] By this is meant the large change in P.D. when 0.01 M KCl is replaced by 0.001 M KCl or by 0.01 M NaCl (the latter is called for convenience the potassium effect).

[6] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

[7] Since the effect of K^+ predominates the other cations are omitted from the discussion. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

allows K^+ to migrate outward in the form of a moving boundary.[8] This destroys the concentration gradient of K^+ across Y and produces a loss of P.D.

On reaching X (when X is sensitive to K^+) K^+ will set up an outwardly directed (positive) potential, causing the curve to fall and producing the p movement of the first peak. This movement will last until K^+ reaches the outer surface of X and thus diminishes the concentration gradient of K^+ across X.

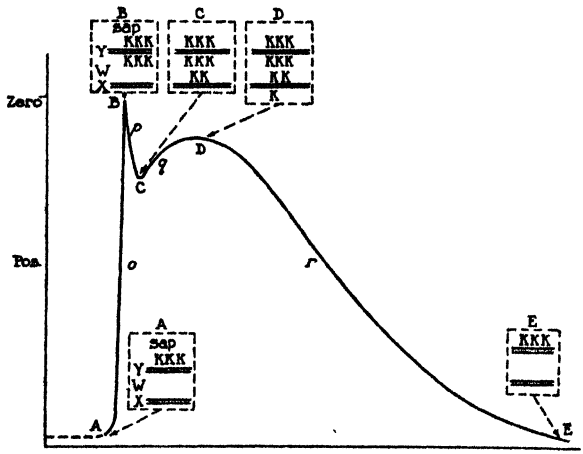

FIG. 1. The unbroken line shows changes in P.D. during the action current in *Nitella*, supposedly due to the outward movement of potassium. The broken line shows the P.D. in the resting state, before the outward movement of potassium begins.

In the diagrams the symbol K denotes the outwardly moving potassium (reduction in concentration is shown by reduction in the number of symbols). Each stage of its progress is marked by a change in P.D.: for example, in Diagram A the observed P.D. is due to the relatively high concentration of potassium at the inner surface of Y; in Diagram B we see that potassium has reached the outer surface of Y and in consequence the P.D. has disappeared.

The duration of the action current is usually about 15 seconds.

The duration of this downward movement will depend on the speed with which K^+ moves across X. Its magnitude will depend on the sensitivity of X to K^+, *i.e.* on the mobility ratio[9] $u_K \div v_{Cl}$, and on the partition coefficient S_K (S_K = concentration of K^+ in the non-aqueous protoplasmic surface $\div$ concentration of K^+ in the adjacent aqueous solution).

It will be larger when the moving boundary is sharp for then the concen-

[8] Regarding moving boundary see MacInnes, D. A., and Longsworth, L. G., *Chem. Rev.*, 1932, **11**, 171.

[9] *Cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

tration gradient of K^+ across X will be larger. If the front of the moving boundary becomes more diffuse the concentration of K^+ as it strikes X will diminish and if it scarcely exceeds the concentration of K^+ already present in W the p movement will practically disappear.

This may explain such a curve as is seen[10] in Fig. 2 which may occur even when X is sensitive to K^+. Such curves are occasionally found, especially in cells exposed to 0.01 M NaCl[11] which may increase the protoplasmic motion and render the moving boundary more diffuse. In such cases it is no longer possible to distinguish sharply between the fall of the curve due to the p movement and the fall due to the r movement (*i.e.* to the recovery which is presumably due to the movement of K^+ back into the sap). In such cases the only evidence of the p movement lies in a sudden change in the course of the curve, as in Fig. 2.

If the outer protoplasmic surface X loses part of its sensitivity to K^+ the p movement will fall off in consequence.

When X has lost its sensitivity to K^+ (as shown by the absence of the potassium effect[12]) there is no longer any reason to expect any abrupt change in the course of the curve.[13]

FIG. 2. Action curve in an unleached cell of *Nitella* with normal potassium effect; *i.e.*, with the outer protoplasmic surface sensitive to K^+. The first peak is missing, presumably because the outwardly moving K^+ has a diffuse rather than a sharp boundary.

The spot recorded, D, is in contact with 0.0001 M NaCl, it is connected through the recording galvanometer to a spot F which is in contact with 0.01 M KCl and consequently has a constant P.D. approximately at zero. Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 10 days in Solution A at 15 ± 1°C. The record was made at 22°C. (electrical stimulation). Vertical marks 5 seconds apart.

[10] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) for several days.

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 541).

[11] (*Cf.* Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, **22**, 91. It is here suggested that increased conductivity of the protoplasm may tend to produce single peaks.

[12] *I.e.* when there is no change in P.D. on replacing 0.01 M KCl by 0.01 M NaCl.

[13] At the start of the spike there may be an abrupt rise of the curve due to electrical leakage from the stimulating electrodes.

FIG. 3. Action curve in a cell of *Nitella* which has lost its potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl and was connected through the recording galvanometer with another spot, *F*, in contact with 0.01 M NaCl (the P.D. of the latter remained constant as evidenced by the record of another spot *E* not shown here). In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 8 days in Solution A, then for 5 days in distilled water: the temperature was 15 ± 1°C. The record was made at 23°C. (electrical stimulation). Vertical marks 5 seconds apart.

FIG. 4. Action curve in a normal cell of *Chara* which shows no potassium effect; *i.e.*, the outer protoplasmic surface is not sensitive to K^+.

The spot recorded, *D*, was in contact with 0.001 M KCl: it was connected through the recording galvanometer with a spot *F* killed by chloroform and having in consequence a P.D. of zero. Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 2 days in Solution A at 15 ± 1°C. The record was made at 24°C. Vertical marks 5 seconds apart.

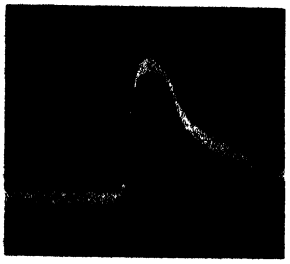

FIG. 5. Action curve in a cell of *Nitella* which has lost the potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl: it was connected through the recording galvanometer with another spot *F* in contact with 0.01 M NaCl which had a constant P.D. during the record as evidenced by the record of another spot *E* (not shown here). In consequence the action curve is monophasic.

The cell was freed from neighboring cells and kept for 12 days in Solution A and then placed in distilled water for 7 days: the temperature was 15 ± 1°C. The record was made at 22°C. Vertical marks 5 seconds apart.

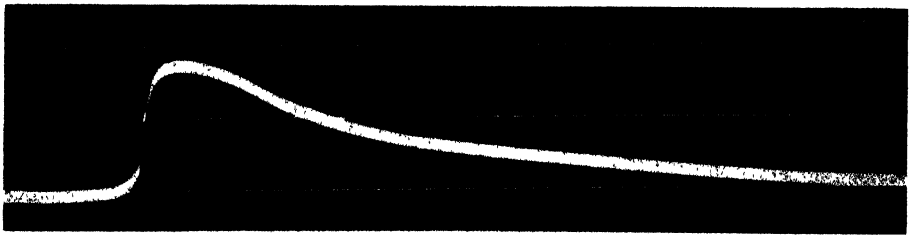

FIG. 6. Action curve in a cell of *Nitella* which has lost the potassium effect (*i.e.* the outer protoplasmic surface has become insensitive to K^+) as the result of leaching in distilled water.

The spot recorded, *D*, was in contact with 0.01 M NaCl: it was connected through the recording galvanometer to another spot *F* in contact with 0.01 M NaCl, which had a constant P.D. during the record, as evidenced by the record of another spot *E* (not shown here). Hence the action curve is monophasic.

The cell was freed from neighboring cells and kept in distilled water for 6 days at 15 ± 1°C. The record was made at 23°C. Heavy vertical marks 5 seconds apart.

Under these circumstances we expect only curves with rounded tops,[14, 15]

[14] Since there is no *p* movement there is no *q* movement.

[15] This applies to action curves in which the P.D. is largely lost and the spike or *o* movement follows the usual course and goes nearly to zero. When only a relatively small loss of P.D. occurs rounded tops may occur even when *X* is sensitive to K. Such action curves are regarded as abnormal and probably involve only a small outward movement of K^+ which may not reach *X* at all or only to a slight extent. See Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369 (Fig. 12 b).

such as are actually observed[16] under these conditions (Fig. 3). Fig. 3 may be compared with the normal curve[17] of *Chara* (Fig. 4).

The course of recovery may be shorter than is usually observed in *Nitella* but this is not always the case, as is evident from Figs. 5 and 6.

These facts strongly support the suggestion previously made[18] that K^+ plays an important rôle in the action curve.

SUMMARY

In *Nitella* the action curve has two peaks, apparently because both protoplasmic surfaces (inner and outer) are sensitive to K^+.

Leaching in distilled water makes the outer surface insensitive to K^+. We may therefore expect the action curve to have only one peak. This expectation is realized.

The action curve thus obtained resembles that of *Chara* which has an outer protoplasmic surface that is normally insensitive to K^+.

The facts indicate that the movement of K^+ plays an important part in determining the shape of the action curve.

[16] In some cases the potassium effect may be restored by pressure on the cell (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18,** 687) or by an action current (Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934-35, **18,** 681). Hence a cell showing an action curve with one peak may sometimes show a potassium effect when tested later on.

When a cell is tested for the potassium effect with negative result but when subsequently stimulated shows a tendency to form a second peak this may be due to the fact that the action current tends to restore the potassium effect and consequently the double peak.

[17] After the spike the curve in Fig. 4 falls below the original level (positive after potential) and then rises. This is not a constant feature of *Chara* and it may also occur in *Nitella*.

In *Chara* the chief seat of the P.D. appears to be (as in *Nitella*) the inner protoplasmic surface *Y* which is presumably sensitive to K^+ although the outer surface, *X*, is not. Leached *Nitella* resembles *Chara* in that *Y* is sensitive to K^+ but *X* is not.

[18] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18,** 215.

EFFECTS OF GUAIACOL AND HEXYLRESORCINOL IN THE PRESENCE OF BARIUM AND CALCIUM

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Höber[1] holds that alkaline earths affect the colloids of protoplasm so as to inhibit the depolarizing effect of K^+. Guttman[2] states that they prevent the depolarizing action of various organic substances.

These conclusions, based on experiments with muscle and nerve, have been tested on *Nitella*. In a previous paper it is shown that in some cells the effect of K^+ is partly inhibited[3] by Ca^{++} when the concentration of K^+ is 0.01 M but not when it is 0.1 M. In other cells no such inhibition occurs. The present paper shows that Ca^{++} and Ba^{++} do not inhibit the depolarizing effects of guaiacol and hexylresorcinol.

The depolarizing action of guaiacol has been described in previous papers.[4] The normal positive[5] P.D. of about 100 mv. decreases (*i.e.*, changes in a negative direction) when guaiacol is applied and if the concentration is not too high the process is reversible.

In order to test the effect of Ca^{++} on this process the following experiments were made.[6]

In all the experiments two spots on the same cell, *D* and *E*, were connected through the recording galvanometer to a spot *F* (which was in

[1] Höber, R., and Strohe, H., *Arch. ges. Physiol.*, 1929, **222**, 71. Höber, R., Andersh, M., Höber, J., and Nebel, B., *J. Cell. and Comp. Physiol.*, 1939, **13**, 195.

[2] Guttman, R., *J. Gen. Physiol.*, 1939–40, **23**, 343.

[3] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938–39, **22**, 139.

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1938–39, **22**, 417; 1939–40, **23**, 171.

[5] The P.D. is called positive when the positive current tends to flow from the sap across the protoplasm to the external solution.

[6] The cells, after being freed from neighboring cells, stood in the laboratory at 15° ± 1°C. in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87) for several days. They belonged to Lot B (*cf.* Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937–38, **21**, 541). Unless otherwise stated the changes here recorded were reversible and there were no signs of injury.

contact with 0.01 M KCl and in consequence had a P.D. which remained constant, approximately at zero, during the experiment).

In one set of cells the spot *D* was at first in contact with 0.01 M NaCl: when this was replaced by 0.01 M NaCl + 0.02 M guaiacol there was a loss[7] of P.D. amounting to 103 ± 11.9 mv. (6 observations on 6 cells). When 0.01 M NaCl in contact with *E* was replaced by 0.01 M $CaCl_2$ there was a loss[7] of P.D. amounting to 16.5 ± 1 mv. (6 observations on 6 cells). Then 0.01 M $CaCl_2$ was replaced by 0.01 M $CaCl_2$ + 0.02 M guaiacol which entailed a further change of P.D. in a negative direction[8] amounting[4] to 115 ± 12.5 mv. (5 observations on 5 cells).

The experiment was repeated on another set of cells, using Ba^{++} in place of Ca^{++}. The result was similar. Addition of 0.02 M guaiacol to 0.01 M NaCl at *D* caused a change of P.D. of 108 ± 11.9 mv. in a negative direction (5 observations on 5 cells). Replacing 0.01 M NaCl by 0.01 M $BaCl_2$ at *E* caused a rapid loss of P.D. amounting to 25 ± 2.3 mv. (5 observations on 5 cells). Replacing 0.01 M $BaCl_2$ by 0.01 M $BaCl_2$ + 0.02 M guaiacol caused a change of P.D. in a negative direction amounting to 107 ± 6.6 mv. (5 observations on 5 cells).

Experiments with hexylresorcinol[9] gave similar results. For example, a set of experiments was made in which 0.01 M NaCl at *D* was replaced by 0.01 M NaCl + 0.0003 M hexylresorcinol, giving a change[10] of P.D. in a negative direction of 104 ± 10.4 mv. (6 observations on 6 cells). When 0.01 M $CaCl_2$ at *E* was replaced by 0.01 M $CaCl_2$ + 0.0003 M hexylresorcinol the change of P.D. was 106 ± 10.5 mv. (6 observations on 6 cells).

In another set of cells 0.001 M NaCl was placed at *D*. When this was replaced[11] by 0.015 M NaCl there was a loss of P.D. of 30 ± 1.2 mv. (8 observations on 8 cells). This solution was then replaced by 0.015 M NaCl + 0.0003 M hexylresorcinol, causing a further loss of 79 ± 7.4 mv. (8 observations on 8 cells).

Then 0.001 M NaCl at *E* was replaced by 0.001 M NaCl + 0.01 M $BaCl_2$ (thus making the osmotic pressure about the same as that of 0.015 M NaCl

[7] This took place in a few seconds, indicating that the effect was chiefly at the outer protoplasmic surface.

[8] This was a gradual change in a negative direction (depolarization) involving an action current in some cases.

[9] The hexylresorcinol was kindly donated by the firm of Sharp and Dohme, Glenolden, Pennsylvania.

[10] Osterhout, W. J. V., *J. Gen. Physiol.*, 1939–40, **23**, 569.

[11] The object of this was to make the osmotic pressure about the same as in the subsequent experiment at *E*.

at *D*): this caused a loss of P.D. of 45 ± 2.8 mv. (8 observations on 8 cells). When this was replaced by 0.001 M NaCl + 0.01 M $BaCl_2$ + 0.0003 M hexylresorcinol there was a further change of P.D. in a negative direction amounting to 106 ± 3.5 mv. (8 observations on 8 cells).

It is evident that the depolarizing effect of guaiacol and of hexylresorcinol is not inhibited by Ca^{++} or Ba^{++}. It is possible that this may be due to the fact that Ca^{++} and Ba^{++} do not penetrate to the inner protoplasmic surface which is the chief seat[12] of the P.D. and which is reached by guaiacol and hexylresorcinol.

The effects of these organic depressants come on slowly, indicating that they penetrate gradually to the inner protoplasmic surface. But when NaCl is followed by $CaCl_2$ or by $BaCl_2$ the change in P.D. is immediate, indicating that their effects are chiefly at the outer protoplasmic surface.

SUMMARY

Guaiacol was applied at two spots on the same cell of *Nitella*. At one spot it was dissolved in 0.01 M NaCl, at the other in 0.01 M $CaCl_2$ or $BaCl_2$. The effect was practically the same in all cases, *i.e.* a similar change of P.D. in a negative direction, involving a more or less complete loss of P.D. (depolarization).

When hexylresorcinol was used in place of guaiacol the result was similar.

That Ca^{++} and Ba^{++} do not inhibit the effect of these organic depolarizing substances may be due to a lack of penetration of Ca^{++} and Ba^{++}. The organic substances penetrate more rapidly and their effect is chiefly on the inner protoplasmic surface which is the principal seat of the P.D.

[12] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

THE ELECTRICAL CONDUCTANCE OF SUSPENSIONS OF ELLIPSOIDS AND ITS RELATION TO THE STUDY OF AVIAN ERYTHROCYTES*

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I

INTRODUCTION

The electrical conductance of a colloidal suspension is a function not only of the individual conductances of the particles and the suspending medium, and of the fraction of the volume occupied by the particles, but also of the shape of the particles, their orientation, and at high concentrations, because of electrical interaction between particles, their array. In the cases where the equations have been developed the electrical conductance measurements on suspensions can provide information either on volume, symmetry, or conductance of the colloidal particles if the values of the other variables are known.

Maxwell (1) derived the equation

$$\frac{(k/k_1) - 1}{(k/k_1) + 2} = \rho \frac{(k_2/k_1) - 1}{(k_2/k_1) + 2} \tag{1}$$

for homogeneous suspensions of non-polarizable spheres where k, k_1, and k_2, are specific conductances of suspension, medium, and particles respectively and ρ is the fraction of the total volume occupied by the suspended phase. The equation was experimentally verified for fat globules in cream by Fricke and Morse (2). Parallel equations for the dielectric constant of suspensions have been experimentally verified by Millikan (3) for emulsions of water in benzene and chloroform.

In order to apply the method to suspensions of non-spherical particles

* This work was done in connection with some problems that arose in the study of the selective invasion of immature red blood cells by parasites in avian malaria and was supported by a grant from the International Health Division of The Rockefeller Foundation to Robert Hegner.

Fricke (4) considered the case of spheroids and expressed his results in the form

$$k = k_1 + \frac{1/3\rho}{1-\rho} \sum^{\alpha=a,b,c} \frac{2(k_2 - k)}{2 + abcL_\alpha(k_2/k_1 - 1)} \tag{2}$$

where ρ, k, k_1, and k_2 are the same as above, a, b, and c are the axial lengths of the ellipsoid, and L_a, L_b, and L_c are integrals which are functions of a, b, and c and are dependent not upon the absolute magnitudes of these quantities but on the ratios, a/c and b/c. Equation (2) reduces to the form

$$\frac{(k/k_1) - 1}{(k/k_1) + x} = \rho \left[\frac{(k_2/k_1) - 1}{(k_2/k_1) + x} \right] \tag{2'}$$

in which x, the form factor, is a function of the axial ratio of the particles and of the conductance ratio (k_2/k_1). Choosing as his model the oblate spheroid he showed that the conductance of suspensions of the biconcave discoidal red cells of mammals closely fitted his equations when a suitable value for the axial ratio of the cells was chosen. Due, however, to deviations of his model from the actual form of the cell the axial ratio which gave the best results in determining ρ differed slightly from the ratio obtained by direct measurement.

To make the method more widely applicable we have extended Fricke's development and have obtained solutions for a more general model, an ellipsoid with three axes different which rather closely approximates the normal shape of the avian erythrocyte. For the case most often met with in practice, k_2 is zero. The equation for ellipsoids assumes the form

$$\rho = \frac{(r/r_1) - 1}{(r/r_1) - 1 + f} \tag{3}$$

where r/r_1 is the ratio of the specific resistance of the suspension to that of the medium, ρ is as before the fraction of the volume occupied by the particles, and f is a factor which depends upon the axial ratios of the ellipsoids. Tables of f for various values of the axial ratios will be presented.

It was observed in confirmation of previous workers that marked changes in conductance occur when the suspension is stirred. The silky reflection pattern from the surface of a suspension swirled in a beaker suggested that the motion of the liquid orients the particles. Orientation was confirmed by light transmission measurements in a specially designed flow cell. We have therefore considered in the theory the effect of orientation upon the electrical conductance of the suspension. Conductivity cells were designed in which the suspension was allowed to flow during the measurements.

Changes in the direction predicted by the theory were observed and the effect shown to be a function of the degree of asymmetry of the particles. When the particles were converted to the spherical form the flow effect, as would be expected, disappeared.

Tables showing the dependence of the form factor, f, upon the axial ratios of the particles in random and four types of directed orientation are presented. With properly designed conductivity cells it should be possible to extend the method to shape studies of submicroscopic particles.

II

Theory

Fundamental Equation

Following Fricke's derivation which neglects the presence of ions inside and outside the particle and also neglects the electrical interaction between the particles themselves, Laplace's equation is used to obtain formally the potential inside and outside the particle due to charges on its surface. By superposition of these potentials upon that of the external field the electric force can be expressed as a function of the volume fraction, ρ, occupied by the particles, the axial ratios, the applied potential V, and the conductances of the particle and medium. For the case in which the a axis of the ellipse is oriented parallel to the external field the equation

$$(F/V)(1-\rho) + \frac{2(F/V)\rho}{2 + abcL_a[(k_2/k_1) - 1]} = 1 \tag{4}$$

results, where L_a is the integral

$$\int_0^\infty \frac{d\lambda}{(a^2+\lambda)\sqrt{(a^2+\lambda)(b^2+\lambda)(c^2+\lambda)}}$$

and F is the electrical force. By applying Ohm's law the additional relation involving F/V and the other quantities is obtained:

$$k = k_1 + \rho\left(\frac{2k_1[(k_1/k_2) - 1]F/V}{2 + abcL_a[(k_2/k_1) - 1]}\right). \tag{5}$$

Eliminating F/V between the equations (4) and (5) we get the final result

$$k = k_1 + \left(\frac{\rho}{1-\rho}\right)\left(\frac{2(k_2 - k)}{2 + abcL_a[(k_2/k_1) - 1]}\right) \tag{6}$$

In the corresponding equations for the cases in which the axes b and c are parallel to the electric field the integral L_a is replaced by L_b and L_c which are of the same form.

For random orientation averaging is accomplished by assuming that one-third of the particles is oriented in each direction. It follows that

$$k = k_1 + \frac{1/3\rho}{1-\rho} \sum^{\alpha=a,b,c} \frac{2(k_2 - k)}{2 + abcL_\alpha[(k_2/k_1) - 1]} \tag{7}$$

Evaluation of the Integrals

In order to apply these equations the three integrals

$$L_a = \int_0^\infty \frac{d\lambda}{(a^2+\lambda)^{3/2}(b^2+\lambda)^{1/2}(c^2+\lambda)^{1/2}}$$

$$L_b = \int_0^\infty \frac{d\lambda}{(b^2+\lambda)^{3/2}(c^2+\lambda)^{1/2}(a^2+\lambda)^{1/2}}$$

$$L_c = \int_0^\infty \frac{d\lambda}{(c^2+\lambda)^{3/2}(a^2+\lambda)^{1/2}(b^2+\lambda)^{1/2}}$$

must be evaluated. This is equivalent to evaluating any one of the L_α integrals, let us say L_a, for the three cases in which a takes successively the values of the three axes of the ellipse, and b and c take the values of the other two axes.

Letting $\lambda = (1/y) - a^2$ the integral becomes when simplified

$$= \int_1^{1/a^2} \frac{y^{1/2}dy}{\sqrt{[(b^2-a^2)y+1][(c^2-a^2)y+1]}}. \tag{8}$$

With the substitution $y = \dfrac{-\psi^2}{b^2-a^2}$ we obtain eventually

$$L_a = \frac{2}{(a^2-b^2)^{3/2}} \int_0^{\sqrt{a^2-b^2}/a} \frac{\psi^2 d\psi}{\sqrt{(1-\psi^2)(1-q^2\psi^2)}} \tag{9}$$

where

$$q^2 = (c^2 - a^2)/(b^2 - a^2).$$

Rearranging we get

$$L_a = \frac{2}{q^2(a^2-b^2)^{3/2}} \left[\underbrace{\int_0^{\sqrt{a^2-b^2}/a} \frac{d\psi}{\sqrt{(1-\psi^2)(1-q^2\psi^2)}}}_{\text{i}} - \underbrace{\int_0^{\sqrt{a^2-b^2}/a} \sqrt{\frac{1-q^2\psi^2}{1-\psi^2}}\, d\psi}_{\text{ii}} \right] \tag{10}$$

The integrals *i* and *ii* are standard elliptic integrals of the first and second kind respectively. To convert them to the forms in which they are most frequently tabulated[1] let $\sin\varphi = \psi$

$$L_a = \frac{2}{q^2(a^2-b^2)^{3/2}}\left[\int_0^{\sin^{-1}[(a^2-b^2)^{1/2}/a]} \frac{d\varphi}{\sqrt{1-q^2\sin^2\varphi}} - \int_0^{\sin^{-1}[(a^2-b^2)^{1/2}/a]} \frac{d\varphi}{\sqrt{1-q^2\sin^2\varphi}}\right]$$

or

$$L_a = \frac{2}{(c^2-a^2)(a^2-b^2)^{1/2}}\left[F\left(\sqrt{\frac{c^2-a^2}{b^2-a^2}}, \sin^{-1}\frac{\sqrt{a^2-b^2}}{a}\right) - E\left(\sqrt{\frac{c^2-a^2}{b^2-a^2}}, \sin^{-1}\frac{\sqrt{a^2-b^2}}{a}\right)\right] \tag{11}$$

Case I, $a > c > b$

Equation (11) is real only for the case in which q is positive and can only be evaluated when $q < 1$; *i.e.*, $a < c < b$ or $a > c > b$. Of these two only the second case can be evaluated directly from the tables, for when $a < c < b$ the upper limit of the integrals becomes imaginary. Since b and c are interchangeable there remain only two other cases to be evaluated, namely $a < c < b$ and $b < a < c$.

Case II, $a < c < b$

For $a < c < b$ let $i\varphi = \psi$ in equation (10). The equation becomes

$$L_a = \frac{2}{q^2(b^2-a^2)^{3/2}}\left[\underbrace{\int_0^{\sqrt{(b^2-a^2)}/a} \frac{\sqrt{1+q^2\varphi^2}}{\sqrt{1+\varphi^2}}\,d\varphi}_{\text{i}} - \underbrace{\int_0^{\sqrt{(b^2-a^2)}/a} \frac{d\varphi}{\sqrt{(1+\varphi^2)(1+q^2\varphi^2)}}}_{\text{ii}}\right] \tag{12}$$

The second integral (ii) in equation (12) is equivalent to

$$F\left[l, \tan^{-1}\left(\sqrt{b^2-a^2}/a\right)\right]$$

where l is given by $l^2 = 1 - q^2 = (b^2 - c^2)/(b^2 - a^2)$ when $0 < l < 1$. The requirement $0 < l < 1$ is met when $a < c < b$.

[1] Pierce, B., A short table of integrals, Boston, Ginn and Co., 3rd revised edition, 1929.

To evaluate the first integral in equation (13) we make the substitution $\varphi = \tan\psi$ and obtain

$$(12)_i = \int_1^{\tan^{-1}(\sqrt{b^2-a^2}/a)} \frac{\sqrt{1 - q^2 \sin\psi}}{\cos^2\psi}\, d\psi$$

which has been evaluated,[2]

$$\begin{aligned}(12)_i = (\sqrt{b^2 - a^2}/a)\sqrt{1 - l^2 \sin^2 (\tan^{-1} \sqrt{a^2 - b^2}/a)} \\ + F[l, \tan^{-1} (\sqrt{b^2 - a^2}/a)] - E[l, \tan^{-1} (\sqrt{b^2 - a^2}/a)]\end{aligned} \tag{13}$$

where

$$l^2 = 1 - q^2 = (b^2 - c^2)/(b^2 - a^2)$$

Case III, $b < a < c$

The third case to be solved is $b < a < c$. We return to equation (10) and make the following substitutions and transformations

$$m^2 = -q^2, \qquad \sqrt{a^2 - b^2}/a = u, \quad \text{and} \quad \psi = \sqrt{1 - t^2}$$

The first integral of (10) becomes

$$(10)_i = \int_1^{\sqrt{1-u^2}} \frac{-dt}{\sqrt{(1 - t^2)(1 + m^2 - m^2 t^2)}} \tag{14}$$

or after rearrangement

$$\begin{aligned}(10)_i = \frac{1}{\sqrt{1 + m^2}} \int_0^1 \frac{dt}{\sqrt{(1 - t^2)(1 - t^2 m^2)/(1 + m^2)}} \\ - \frac{1}{\sqrt{1 + m^2}} \int_0^{\sqrt{1-u^2}} \frac{dt}{\sqrt{(1 - t^2)(1 - t^2 m^2)/(1 + m^2)}}\end{aligned} \tag{15}$$

It follows immediately that in standard form

$$(10)_i = \sqrt{\frac{(b^2 - a^2)}{(b^2 - c^2)}} \left[F\left(\frac{\sqrt{a^2 - c^2}}{\sqrt{b^2 - c^2}}, \pi/2\right) - F\left(\frac{\sqrt{a^2 - c^2}}{\sqrt{b^2 - c^2}}, \sin^{-1} b/a\right)\right]$$

Integration of $(10)_{ii}$ is achieved by the substitution $m^2 = -q^2$, and the transformation $\cos\varphi = \psi$. The integral, $(10)_{ii}$, becomes

$$\sqrt{1 + m^2} \int_{\cos^{-1}(\sqrt{a^2-b^2}/a)}^{\pi/2} \sqrt{[(1 - m^2)/(1 + m^2)] \sin^2\varphi}\, d\varphi$$

or

$$(10)_{ii} = \sqrt{\frac{b^2 - c^2}{b^2 - a^2}} \left[E\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \pi/2\right) - E\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \cos^{-1} \frac{\sqrt{a^2 - b^2}}{a}\right)\right] \tag{16}$$

[2] Potin, L., Formules et tables numerique, Paris, G. Doin, Gauthier, Villars and Co., 1925, 712.

Summing up, the three integrals necessary for calculations with equations (7), (6), or (8) are given below in terms of the standard elliptic integrals of the first and second kind $F(q, \varphi)$ and $E(q, \varphi)$, respectively

$$a > c > b \quad L_a = \frac{2}{(a^2 - c^2)(a^2 - b^2)^{1/2}}\left[F\left(\sqrt{\frac{c^2 - a^2}{b^2 - a^2}}, \sin^{-1}\frac{\sqrt{a^2 - b^2}}{a}\right) - E\left(\sqrt{\frac{c^2 - a^2}{b^2 - a^2}}, \sin^{-1}\frac{\sqrt{a^2 - b^2}}{a}\right)\right]$$

$$a < c < b \quad L_a = \frac{2}{(c^2 - a^2)(b^2 - a^2)^{1/2}}\left[\frac{\sqrt{b^2 - a^2}}{a}\sqrt{1 - \frac{b^2 - c^2}{b^2 - a^2}\sin^2\left(\tan^{-1}\frac{\sqrt{b^2 - a^2}}{a}\right)} - E\left(\sqrt{\frac{b^2 - c^2}{b^2 - a^2}}, \tan^{-1}\frac{\sqrt{b^2 - a^2}}{a}\right)\right]$$

$$b < a < c \quad L_a = \frac{2\sqrt{c^2 - b^2}}{(c^2 - a^2)(a^2 - b^2)}\left[E\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \pi/2\right) - E\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \sin^{-1} b/a\right)\right] - \frac{2}{(c^2 - a^2)(c^2 - b^2)^{1/2}}\left[F\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \pi/2\right) - F\left(\sqrt{\frac{a^2 - c^2}{b^2 - c^2}}, \sin^{-1} b/a\right)\right]$$

The values of $abc\, L_\alpha$ for different sets of axial ratios and for the three orientations in the external field are given in Table I. Instead of absolute values of the absolute length we use the axial ratios referred to the smallest axis as unity.

Ellipsoids of Revolution

For ellipsoids of revolution (rods and discs) we arrive at a similar set of equations. Considering the prolate spheroid with axes a, b, c and with $b = c$ and $a > b$ we have

$$k = k_1 - \frac{1/3\rho}{1 - \rho}\sum^{\alpha=a,b,b} \frac{2}{2 - ab^2 L_\alpha} \tag{17}$$

The two integrals to be obtained are

$$L_a = \int_0^\infty \frac{d\lambda}{(a^2 + \lambda)^{3/2}(b^2 + \lambda)} \tag{18}$$

$$L_b = \int_0^\infty \frac{d\lambda}{(b^2 + \lambda)^{3/2}(a^2 + \lambda)^{1/2}} \tag{19}$$

Integrating by parts

$$L_a = \frac{2}{ab^2} - 2\int_0^\infty \frac{d\lambda}{(a^2 + \lambda)^{1/2}(b^2 + \lambda)^2} = \frac{2}{ab^2} - 2L_b$$

Let $b^2 + \lambda = \delta$, then

$$L_b = \int_{b^2}^{\infty} \frac{d\delta}{(\sqrt{a^2 - b^2 + \delta})\delta^2} = \frac{a}{b^2(a^2 - b^2)} + \frac{1}{2(a^2 - b^2)^{3/2}} \log \frac{a - \sqrt{a^2 - b^2}}{a + \sqrt{a^2 - b^2}} \tag{20}$$

For the oblate spheroid, the approximate model of the mammalian red cell, the axes are a, b, c; $b = c$ and $a < b$. The integral (20) assumes the form

$$\int_{b^2}^{\infty} \frac{d\delta}{\delta^2\sqrt{a^2 - b^2 + \delta}} = \frac{a}{b^2(a^2 - b^2)} - \frac{1}{(b^2 - a^2)^{3/2}} \tan^{-1} \sqrt{\frac{a^2}{b^2 - a^2}} + \frac{\pi}{2(b^2 - a^2)^{3/2}} \tag{21}$$

Values of L_a for various sets of axial ratios are also given in Table I.

TABLE I

Dependence of $abcL_a$ on the Axial Ratios a/c and b/c

a/c	b/c	c/c	$abcL_a$	$abcL_b$	$abcL_c$
2	2	1	0.472	0.472	1.069
3	2	1	0.318	0.535	1.158
4	2	1	0.222	0.574	1.208
5	2	1	0.175	0.593	1.253
6	2	1	0.136	0.607	1.291
7	2	1	0.110	0.617	1.328
3	3	1	0.360	0.360	1.263
4	3	1	0.277	0.396	1.326
5	3	1	0.217	0.417	1.371
6	3	1	0.167	0.433	1.406
7	3	1	0.138	0.442	1.438
4	4	1	0.297	0.297	1.404
5	4	1	0.233	0.318	1.453
6	4	1	0.191	0.331	1.482
7	4	1	0.157	0.343	1.500
5	5	1	0.250	0.250	1.500
6	5	1	0.211	0.273	1.532
7	5	1	0.176	0.282	1.552

Suspensions of Erythrocytes

The suspensions of ellipsoids used for experimental study were suspensions of the red blood cells of birds in serum and in isotonic solutions of sodium chloride. At the frequencies at which the resistance measurements were made the red cells behave as non-conductors and therefore $k_2 = 0$. The simplified equation for random orientation is

$$k_1 = k + \frac{\rho k f_1}{1 - \rho} = k\left(1 + \frac{\rho f_1}{1 - \rho}\right) \tag{22}$$

where $f_1 = 2/3 \sum^{\alpha=a,b,c} \frac{1}{2 - abcL_\alpha}$ is the form factor. Expressed as electrical resistance instead of conductance

$$\rho = \frac{(r/r_1) - 1}{(r/r_1) - 1 + f_1} \tag{23}$$

where ρ is the same as before and r and r_1 are the resistances of the suspension and medium respectively, measured in the same conductivity cell.

Orientation

The types of orientation of the ellipsoids with respect to the electrodes are shown in Fig. 1.

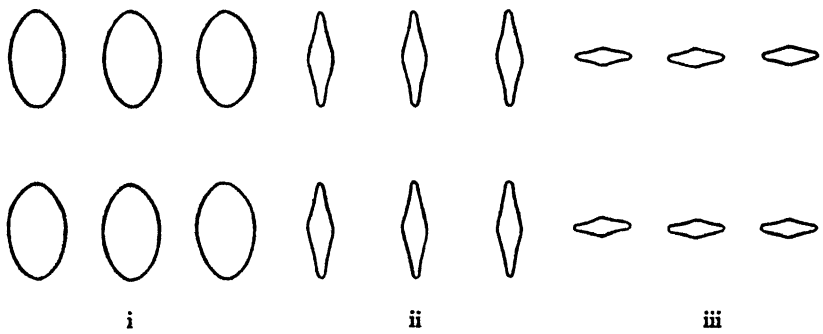

FIG. 1. The three projections of an ellipsoidal red cell with three axes different

When the ellipsoids are oriented at random in the field we take, in effect, the average of the three positions, i, ii, and iii (Fig. 1). If a special orientation is obtained by allowing the blood to flow past the electrodes the type of orientation obtained with respect to the electrodes depends upon the position of the electrodes in the stream. If the arrangement is such that the external field is parallel to the long axis of the particles the effective target area presented by the cells to the lines of electrical force is shown (Fig. 1) in iii the field being directed into the page, or in i or ii when the field is in the plane of the page from top to bottom.

The equation in this case (external field parallel to long axis) is

$$k = k_1 - \frac{2k\rho}{1 - \rho}\left(\frac{1}{2 - abcL_\alpha}\right) \tag{24}$$

Or in the form of equation (23)

$$\rho = \frac{(r/r_1) - 1}{(r/r_1) - 1 + f_2} \tag{25}$$

where

$$f_2 = \frac{2}{2 - abcL_a}$$

when a is taken as the long axis of the ellipsoid.

When the electrodes are arranged so that the long axis of the ellipsoid is perpendicular to the electrical field the target area offered by the cells to the lines of electrical force is the average of i and ii (Fig. 1) if the field is taken as normal to the page. The corresponding equation is

$$2k = 2k_1 - \frac{2k\rho}{1 - \rho} \sum^{a=b,c} \frac{1}{2 - abcL_a} \tag{26}$$

which reduces to

$$\rho = \frac{(r/r_1) - 1}{(r/r_1) - 1 + f_3} \quad \text{where} \quad f_3 = \sum^{a=b,c} \frac{1}{2 - abcL_a} \tag{27}$$

taking b and c as the intermediate and short axes of the ellipsoid respectively.

If the orientation is further restricted so that at the same time either the intermediate or short axis is parallel to the external field the conductance equation again assumes the form of equation (6) with a taken as the axis parallel to the external field.

Application of the conductance equation to suspensions of non-conducting ellipsoids requires the evaluation of the form factor f which is determined solely by the axial ratios of the particles. In Table II are presented the values of the form factors for various combinations of axes and for the various possible orientations of the cells.

III

EXPERIMENTAL

Determination of Volume Fraction and Form Factor

The working equation, $\rho = \dfrac{r/r_1 - 1}{r/r_1 - 1 + f}$, contains four experimentally (5) determinable quantities that can be approached by independent methods. When used to determine the volume concentration of the suspended phase, the appropriate form factor, f, must first be obtained. For spheres the value of f has been deduced and confirmed experimentally (see page 753). The functions for spheroids evaluated by Fricke have been tested only on the non-conducting discoidal red blood cells of mammals which are rough approximations of the spheroidal model. The chief difficulty in working with red cells is that accurate measurements of the short axis of the cell

have not been obtained. Deviations of the cell from the mathematical model occur along the short axis. It is therefore necessary to use an average value for this dimension. Confirmation of the theory for spheroids resides in the fact that when a suitable value for the short axis is chosen a single form factor can be selected which gives a close correspondence between experiment and theory over a wide range of concentrations. The value for the short axis so chosen falls within the limits imposed by direct measurement.

TABLE II

Form Factors for Various Axial Ratios and Orientations

Axial ratios	Random orientation	Long axis parallel to external field, particle free to rotate	Long axis perpendicular to external field, particle free to rotate	Long axis perpendicular, intermediate axis parallel	Long axis perpendicular, short axis parallel	Model
1:1:1	1.500	1.500	1.500	1.500	1.500	Sphere
2:2:1	1.589	1.308	1.729	1.308	2.150	Oblate spheroid
3:2:1	1.643	1.188	1.870	1.364	2.376	Ellipsoid
4:2:1	1.684	1.124	1.964	1.402	2.526	"
5:2:1	1.731	1.096	2.049	1.422	2.676	"
6:2:1	1.776	1.072	2.128	1.436	2.820	"
7:2:1	1.827	1.058	2.211	1.446	2.976	"
3:3:1	1.718	1.220	1.967	1.220	2.714	Oblate spheroid
4:3:1	1.791	1.160	2.106	1.246	2.966	Ellipsoid
5:3:1	1.855	1.122	2.222	1.264	3.180	"
6:3:1	1.911	1.090	2.322	1.276	3.368	"
7:3:1	1.973	1.074	2.422	1.283	3.560	"
4:4:1	1.901	1.174	2.265	1.174	3.356	Oblate spheroid
5:4:1	1.992	1.132	2.422	1.189	3.656	Ellipsoid
6:4:1	2.054	1.104	2.529	1.198	3.860	"
7:4:1	2.097	1.086	2.603	1.207	4.000	"
5:5:1	2.195	1.142	2.572	1.143	4.000	Oblate spheroid
6:5:1	2.183	1.118	2.716	1.158	4.274	Ellipsoid
7:5:1	2.242	1.096	2.815	1.164	4.466	"
2:1:1	1.539	1.210	1.703	1.703	1.703	Rod
3:1:1	1.577	1.122	1.805	1.805	1.805	"
4:1:1	1.597	1.086	1.853	1.853	1.853	"
5:1:1	1.615	1.059	1.894	1.894	1.894	"
6:1:1	1.627	1.045	1.919	1.919	1.919	"
7:1:1	1.633	1.035	1.932	1.932	1.932	"

It was hoped in extending the theory to cover the case of ellipsoids with three axes different that the avian red cell would fit the ellipsoidal model more closely than the mammalian cell fits the spheroidal model and such was found to be the case (see Fig. 2). However, the difficulty in determin-

ing the value of the short axis still exists. Table III shows the range of dimensions of cells in a single sample of blood and the individual variation from bird to bird. The average axial ratios of the cells in the above sample are a/c: b/c: c/c = 4.8:2.9:1 which correspond to a form factor (Table II) of 1.85. This form factor (1.85) is to be compared with the one obtained directly from conductance measurements on the same duck blood.

The form factor, f, and the volume fraction, ρ, can be determined independently by conductance measurements, alone. To accomplish this, a relatively concentrated suspension is taken and r/r_1 is obtained. Then the suspension is diluted by the addition of known amounts of medium. The ratio, r/r_1, is determined for each mixture. Dilution is continued until a volume fraction of about 0.15 is reached. From the data, ρ and f are obtained in the following way. Let ρ' and (r'/r'_1) be the volume fraction

TABLE III

Dimensions of Red Blood Cells of Ducks

Bird	Length		Width		Thickness	
	Average	Extremes	Average	Extremes	Average	Extremes
1	13.7	12.3–15.4	8.0	6.9– 9.1	2.8	2.4–3.0
2	13.9	12.1–14.9	8.1	6.5–10.0	3.1	2.7–3.6
3	13.4	12.9–14.3	8.3	7.8– 9.2	2.8	2.0–3.0
4	13.6	12.5–14.6	8.4	7.6– 9.0	2.7	2.1–2.9

and conductance ratio, respectively, for the most dilute member of the series and ρ and (r/r_1) be the same for any other member of the series. From equation 23 it follows that

$$\rho'/\rho = \rho'\left(\frac{1 - r/r_1 + f}{1 - r/r_1}\right)$$

or

$$v/v' = \rho'\left(1 + \frac{f}{(r/r_1 - 1)}\right) \tag{28}$$

where v' is the total volume to which 1 cc. of the original suspension was diluted in the case of the most dilute member of the series and v is the total volume containing 1 cc. of the original suspension for any other member of the series. The quantities v/v' and r/r_1 are known and experimentally determined, respectively. Then v/v' is plotted against $\frac{1}{r/r_1 - 1}$. A straight line should result of intercept ρ' and slope $\rho' f$.

In Fig. 2 a dilution series on duck erythrocytes in 0.90 per cent NaCl is plotted as outlined above. It may be seen that the first six points fall on a straight line within the experimental error and that the last three points are

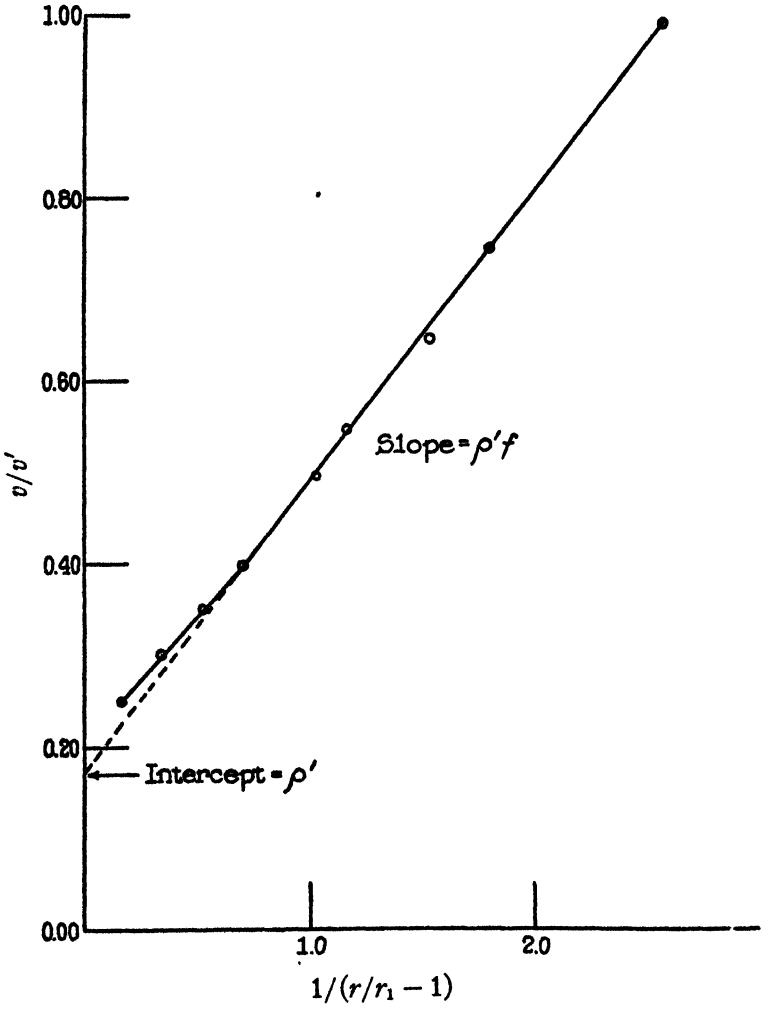

FIG. 2. Dilution series on duck erythrocytes

slightly but definitely above the line. The intercept is 0.172 ± 0.002 and the slope 0.323 ± 0.001. Therefore $\rho' = 0.172 \pm 0.002$ and $f = 1.88 \pm 0.02$. The agreement between this value obtained by conductance measurements, 1.88, and the value predicted by the theory from direct measurements of the cells, 1.85, is satisfactory. The same data are presented in Table IV.

It may be seen that the agreement is good except for the last three points. The deviations at high concentrations are probably caused in part at least by a distortion of the cells as they crowd together. It should be noted that the deviations are opposite in direction to those which would occur if the cell membrane were not completely non-conducting under the conditions of measurement.

Orientation of Cells

When a blood suspension in a cylindrical conductance cell is stirred by a pulsating air jet and then resistance measurements made at 30 second intervals it is found that the resistance slowly rises until a constant value

TABLE IV

Dilution Series of Red Cells of Duck in 0.90 Per Cent NaCl

0.9 per cent NaCl added to 5.0 ml. of suspension	r_s resistance of suspension	r_s/r_m	ρ Calculated from conductance	ρ Calculated from dilution
ml.	*ohms*			
0	5460	6.68	0.732	0.688
1	3200	3.91	0.607	0.573
2	2350	2.88	0.500	0.491
3	1980	2.42	0.436	0.430
5	1620	1.98	0.343	0.344
6	1530	1.87	0.316	0.313
8	1360	1.66	0.259	0.265
10	1280	1.56	0.229	0.229
15	1140	1.39	0.172	0.172

is obtained. The phenomenon is what would be expected to occur if the cells were oriented by stirring and then slowly assumed random orientation after the cessation of stirring.

That orientation occurs is indicated by the silky moiré pattern on the surface of blood as it is swirled in a beaker. It is further demonstrated by consecutive light transmission measurements through a thin flat chamber containing blood at rest and then in laminar flow. The optical cell is mounted in place of the absorption cell in a photoelectric photometer (6). In the dilute suspensions of duck erythrocytes employed the transmission increases 15 to 20 per cent as soon as the suspension is allowed to flow and remains constant during flow. When the flow is stopped the transmission slowly decreases until the original resting value is attained, indicating that the orientation has again become random. The effect is not strikingly dependent upon the wave length.

In order to study the effect of orientation upon conductance similar flow cells were constructed. In the first cell (Fig. 3) coaxial conical electrodes of the Shedlovsky type (7) were fused into a cylindrical tube so that the electrical field would be parallel to the line of flow. In a second conductance (Fig. 4) cell the flow chamber was flattened and the electrodes placed at the sides to make the electrical field perpendicular to the line of flow of the suspension. The chamber actually was identical with that of an Abramson electrophoresis cell (8). When the flow is laminar the particles line up with their long axes parallel to the stream lines, and if the particles are flat the flat surface orients parallel to the wall of the flow chamber. In cell I the effective target area offered by the particles in distorting the electrical field is shown in Fig. 1 (iii) which represents the projection of the oriented cells on the electrode. The corresponding projection for cell II is shown in Fig. 1 (ii).

In Table V are presented the predicted resistances in cells I and II containing suspensions of particles of different shapes in equal volume concentration, oriented at random and in the positions obtained in the flow chambers. The calculations are made

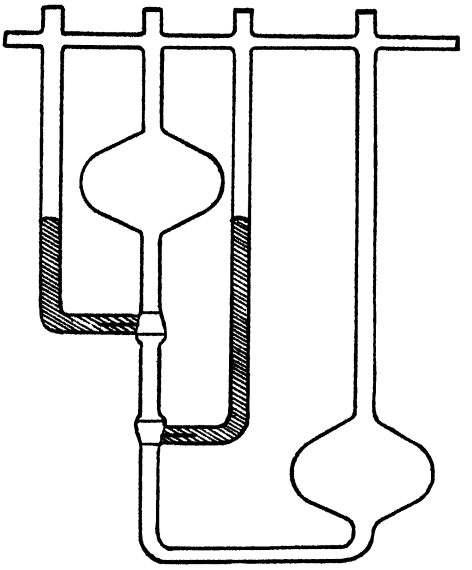

FIG. 3. Cell I—viscometer form of conductance cell for flow measurements with long axis of particles parallel to the electric field.

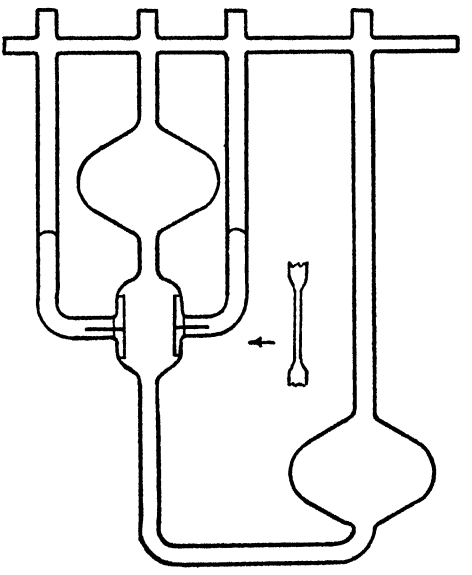

FIG. 4. Viscometer cell II. The orientation of particles is with the long axis perpendicular to the external field and the other two axes are either randomly or explicitly oriented.

for 30 per cent suspensions in which the resistance of the medium is 1000 ohms as directly measured in both conductance cells. It is assumed that the orientation is complete and the lines of electrical force are either parallel or perpendicular respectively to the stream lines at every point.

It is seen that under ideal conditions the resistance measurements of resting and flowing suspensions can provide information on particle shape independently of other methods. The hydrodynamic properties of the flat conductance cell II are such that both spheroidal and ellipsoidal erythro-

TABLE V

Theoretical Effect of Particle Shape and Orientation on the Electrical Resistance of a 30 Per Cent Suspension

Shape	Axial ratio	Resistance of suspension		
		Random	Long axis parallel to field (cell I)	Long axis perpendicular to field, intermediate axis parallel (cell II)
Sphere	1:1:1	2071	2071	2071
Rod	2:1:1	2088	1947	2158
	4:1:1	2113	1894	2223
	6:1:1	2126	1876	2251
Disc (oblate spheroid)	2:2:1	2110	1989	1989
	3:3:1	2165	1951	1951
	4:4:1	2243	1932	1932
	5:5:1	2369	1918	1918
Ellipsoid	4:2:1	2150	1910	2029
	6:2:1	2190	1888	2044
	4:3:1	2196	1926	1963
	6:3:1	2248	1896	1975
	5:4:1	2282	1914	1938
	7:4:1	2327	1894	1946
	6:5:1	2364	1908	1925
	7:5:1	2389	1898	1927

cytes project only their thin edges on the electrodes and suspensions of both therefore decrease in resistance during flow. Rods on the other hand offer a maximum target area to the electrical field under these conditions and correspondingly the resistance of a suspension of rods is increased during flow. It is thus possible to differentiate between platelets and rods.

Flow experiments in the present conductance cells have in the cases studied produced effects in the predicted directions but the magnitude of the effects is less than what would occur if the orientation were complete. Actually the flow cells are not hydrodynamically perfect and the electrical fields are not completely controlled so that the maximum effect is not to be

expected. Data for the various orientation effects are presented in Table VI.

The method may be used to follow experimentally produced shape (9) changes of particles in a suspension. The red blood cells of rabbits suspended in isotonic sodium chloride are approximately discs and the resistance of the suspension decreases during flow. If a small amount of the medium is pipetted off after centrifugation and replaced with an equal volume of dilute rose bengal in isotonic sodium chloride the cells become spherical and, having become completely symmetrical, can no longer be oriented. Correspondingly there is no decrease in resistance when the suspension is allowed to flow. If now the suspension is again centrifuged and some of the medium replaced with dilute serum the effect of the dye is counteracted and the cells again assume the discoidal shape. When the

TABLE VI

Experimental Flow Effects

Particles	Shape	Axial ratios	Random	Flow cell I	Flow cell II	Medium
Duck erythrocytes	Ellipsoid	4.8:2.9:1	1760	1660	1655	1025
Rabbit erythrocytes	Oblate spheroid	4.3:4.3:1	1820	1680	1671	983
E. coli	Rod	4:1:1	987	968	994	790
Cream	Sphere	1:1:1	4521	4528	4529	2360
Spherical red cells	Sphere	1:1:1	1260	1262	1261	830

suspension is allowed to flow its electrical resistance again decreases as it did in the original state

$$\underset{\Delta r_s = -70\Omega}{\text{Discs}} \xrightarrow{\text{rose bengal}} \underset{\Delta r_s = 0}{\text{spheres}} \qquad \text{serum} \qquad \underset{\Delta r_s = -60\Omega}{\text{discs}}$$

When horse erythrocytes are washed in isotonic saline they become almost spheres. If a suspension of such cells is placed in flow cell I and the decrease in resistance during flow is measured a slight effect is obtained corresponding to the slight asymmetry of the cells (random orientation conductance measurements indicate an axial ratio of about 1.7:1.7:1). The cells resume the discoidal shape when serum is added. Correspondingly after the addition of the limiting amount of serum one suddenly begins to get a marked decrease in resistance during flow. When more serum is added one begins to get erratic results due to the extensive rouleaux formation that horse erythrocytes undergo in the presence of sufficient amounts of serum. The flow effect thus provides an objective method for following

shape changes of erythrocytes and should provide an objective means of titrating the antisphering factor of rabbit cells (10).

When erythrocytes are aggregated in rouleaux the effective shape of the suspended particles is that of rods. The theory predicts that in cell II an increase in resistance is obtained during flow of a suspension of rods and such was found to be the case with heparinized horse blood in which all of the erythrocytes are in rouleaux. However the tendency to rouleaux formation is so great that the rouleaux themselves aggregate in irregular forms so that a simple mathematical treatment is impossible. The experiments with horse blood are further complicated by the exceedingly rapid rate of sedimentation.

The conductance measurements were made on a grounded bridge using a vacuum tube audio oscillator and two stages of amplification. Since ratios of resistances rather than absolute magnitudes were required it was sufficient to make the measurements in air in a closed room in which the temperature varied only a few tenths of a degree during a series of measurements. Blood was drawn by heart puncture, defibrinated, and the erythrocytes suspended either in serum or isotonic sodium chloride.

Addendum.—In connection with the conductance of suspensions of oriented rods it is of interest that Curtis and Cole (11) measured the transverse resistance of a single *Nitella* cell and found that the data fitted a modified form of Raleigh's equation (12) for infinite rods oriented with their long axis perpendicular to the electrical field. Raleigh's equation when thrown into the form of equation (3) gives a form factor, f, of 2, which may also be obtained by equation (17) as the limiting value for large axial ratios. Similar equations were applied to muscle and to nerve fibers (13).

SUMMARY

1. The theory of electrical conductance of colloidal suspensions has been extended to cover the case of ellipsoids with three axes different.

2. The results have been applied to suspensions of ellipsoidal erythrocytes of birds.

3. It has been shown that fluctuations in electrical resistance of suspensions of erythrocytes after stirring are due to streaming orientation of the cells.

4. The theory has been extended to cover four cases of orientation and tested experimentally in specially designed flow cells by electrical and optical methods.

5. Application of the flow method to the study of the shape of colloidal particles is discussed.

The authors wish to thank Dr. Theodore Shedlovsky for suggesting the viscometer form for the flow cells and for the use of his shielded bridge for preliminary measurements.

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THE INFLUENCE OF SODIUM CHLORIDE AND TEMPERATURE ON THE ENDOGENOUS RESPIRATION OF B. CEREUS

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I

INTRODUCTION

Analysis of the temperature coefficients of chemical reactions has in certain cases thrown light on the mechanism of the reaction. Attempts have been made to interpret the action of temperature on biological reactions in the same way. Crozier (1924–25) has emphasized the importance of master reactions in controlling the temperature coefficient, while Bělehrádek (1935) has stressed the part played by diffusion, and the viscosity of the medium in which the reaction occurs.

The present paper describes experiments in which the rate of respiration by *B. cereus* has been measured at temperatures within the range 11 to 27°C., at different pHs, and in different concentrations of sodium chloride. The data are then interpreted in terms of the hypotheses advanced by Crozier and Bělehrádek.

II

EXPERIMENTAL PROCEDURE

The material and methods were the same as those used in previous studies on this organism (Ingram 1939*a* and *b*). Buffered suspensions of washed cells of *B. cereus* were mixed with an equal volume of distilled water or salt solution and the rates at which oxygen was consumed by these mixtures were measured in Barcroft differential manometers. The manometers were placed in thermostats, where their temperature was maintained to ±0.05°C.

Two methods were used, for the comparison of the rates of oxygen consumption at different temperatures with those at 25°C. The first method, employed in the experiments at pH 6.0, involved the preparation of two comparable series of suspensions; these were compared at 25°C. as a check on their similarity, after which one series was kept at this temperature, and the other was placed in a thermostat at some other temperature for comparison with the standards. These measurements were in some cases made up to 15 hours after the preparation of the suspensions, by which time the exponential decline of the rate of respiration (with time) was well under way (Ingram, 1939*a*).

Stier and Stannard (1937) have shown that the temperature coefficient of respiration by bakers' yeast does not change when the exponential decline of respiration sets in.

The possibility that *B. cereus* might behave differently was tested by the use of a second method of comparison. One series of suspensions was employed during the period of constant respiration, and alternated between the reference temperature of 25°C. and the temperature of the investigation. Observations were made at pH 6.5 and 7.5 over a considerable range of temperature, following this procedure. This second method has the defect that there may be an appreciable lag in the adjustment of the protoplasm to change of temperature. It was found, however, to give results similar to those obtained by the first method, when 10 minutes were allowed for adjustment to take place.

III

Experimental Data

The equation used by Arrhenius (1889) to express the effect of temperature on the velocity of a chemical reaction is

$$\ln. \frac{k_1}{k_2} = \frac{\mu}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{1}$$

where k_1 and k_2 are the velocity constants at absolute temperatures T_1 and T_2 respectively, R is the gas constant, and μ is a constant characteristic of the reaction which measures the mean energy required to activate the reacting molecules. When applied to a biological reaction proceeding at a constant rate, this equation may be transformed into

$$\mu = 4.6 \frac{\log r_1 - \log r_2}{\left(\frac{1}{T_2} - \frac{1}{T_1}\right)} \text{ calories} \tag{2}$$

where r_1 and r_2 are the rates of the reaction at absolute temperatures T_1 and T_2. Thus if equation (1) is valid, one should obtain a straight line of slope $\mu/4.6$ by plotting $\log r$ against $1/T$.

The data obtained at pH 7.5 and at pH 6.5, over the temperature range 11–27°C., are set out in this way in Figs. 1 and 2 respectively. These figures show that at pH 7.5 or at pH 6.5 the addition of sodium chloride in concentrations between 0.6 M and 1.8 M reduced the rate of respiration considerably, but that the value of μ was not changed. Fig. 3 gives the data obtained over a more restricted range of temperature, 20–25°C., at pH 6.0. In the presence of low concentrations of sodium chloride the rate of respiration was raised, and the value of μ was increased at the same time.

The rates of respiration given on Figs. 1–3 are referred to a common basis, which is the rate of respiration in distilled water at 25°C. taken as 100 units. This corresponds to a Q_{O_2} of about 20. It should be remarked that the

addition of salts to suspensions in phosphate buffers causes them to become slightly more acid, so that the pHs given are only approximate. This has been discussed in an earlier paper (Ingram, 1939 *b*).

The data of Figs. 1 and 2 indicate that the value of μ was probably less the higher the temperature. This was particularly evident at low temperatures. There was no evidence to suggest that the fall was discontinuous. The change in the value of μ with temperature makes it impossible to compare the various values, except when they are calculated over the same interval of temperature. Thus, in order to compare the effect of temperature at pH 6 over the range 20 to 25°C. with that at pH 6.5 and 7.5 over the range 11 to 27°C., it is necessary to resort to some coefficient which has a unique value over a wide range of temperature.

Bělehrádek (1935) has shown that the rate of many biological reactions may be related to temperature by means of the equation

$$r = a \cdot t^b \quad (3)$$

$$i.e.\ \log r = \log a + b \cdot \log t \quad (4)$$

where r is the rate of the reaction at a temperature of t°C. and a and b are constants. This equation may be applied over a wide range of temperature with a single value of b,

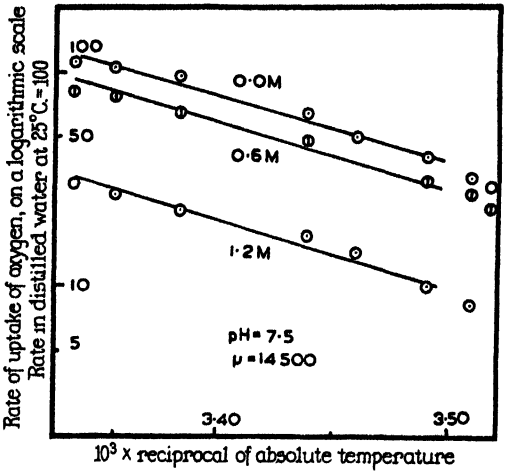

FIG. 1. The rates of respiration at pH 7.5, in the presence of various concentrations of sodium chloride.

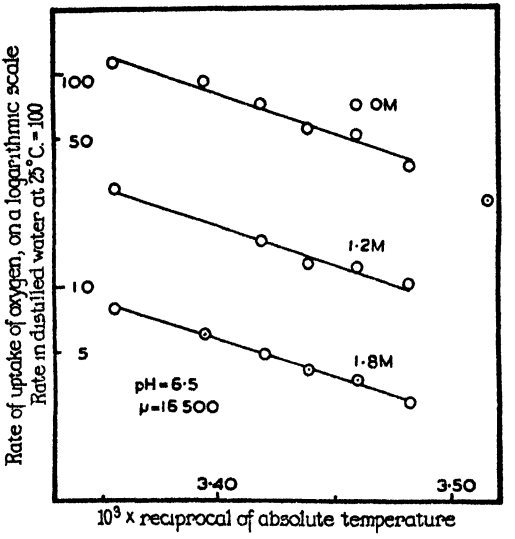

FIG. 2. The rates of respiration at pH 6.5, in the presence of various concentrations of sodium chloride.

and it is therefore suitable for comparing the data referring to pH 6.0 with those obtained at pH 6.5 and 7.5. This is done in Fig. 4 by plotting log r

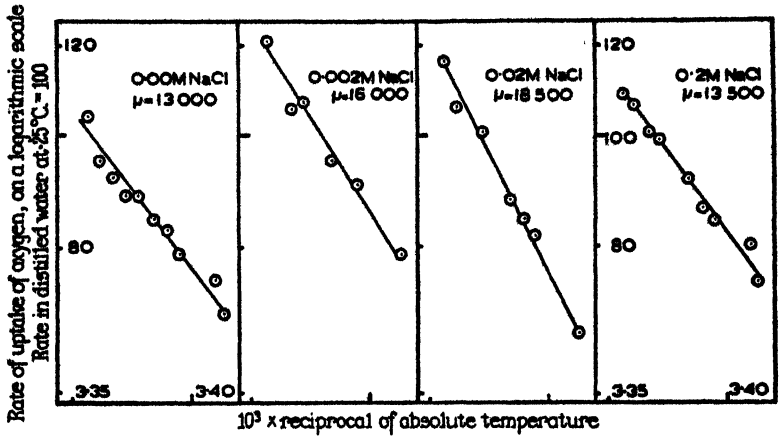

FIG. 3. The rates of respiration at pH 6.0, in the presence of various concentrations of sodium chloride.

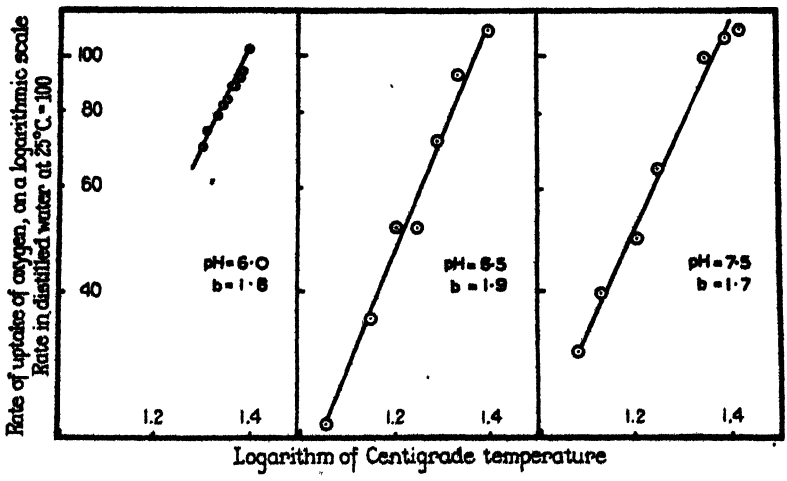

FIG. 4. The rates of respiration in buffer solution only, at different pHs

against log t. The slope of each line represents the value of b. Fig. 4 shows that the temperature coefficient was not much altered by change of pH, but that it was greatest at pH 6.5. The rate of respiration was also greatest at this pH, which is near to the pH of optimum respiration for *B. cereus* (Ingram, 1939*b*).

IV

DISCUSSION

The data presented in the previous section make it clear that the alterations in the temperature coefficient of respiration which are caused by the presence of sodium chloride are related to the effect of the salt on respiration. If the concentration of salt present is greater than 0.2 M the rate of respiration is reduced, and the temperature coefficient is not changed; with concentrations less than 0.2 M the rate of respiration is increased, and the temperature coefficient is raised. Moreover, it appears that if the rate of respiration is varied by alteration of pH, the temperature coefficient is higher, the greater the rate of respiration; in this case, however, the change in temperature coefficient is smaller than that brought about by the addition of small amounts of sodium chloride.

Investigations of this subject are so few that it is impossible to obtain confirmation of these results from earlier studies within the realm of bacterial physiology, but such data as are available from other fields agree fairly well with those above. From the data of Martin (1904) referring to the beat frequency of terrapin heart in potassium chloride solutions, Bělehrádek (1935) decided that the temperature coefficient passed through a maximum, and then declined again with increasing salt concentration. As the most concentrated solution corresponded to about 0.04 M KCl, this behavior is in agreement with that reported at pH 6.0 in section III of the present communication, so far as the relation towards the salt is concerned; but the increase in the temperature coefficient was much greater than has been observed with respiring *B. cereus*. Sherwood and Fulmer (1926) showed for the growth of yeast in a synthetic medium, that the value of μ was increased only slightly, by the presence of a little ammonium chloride in some of the nutrient solutions. The recent work of Bodine and Thompson (1935) is also relevant. These workers have presented measurements of the rate of consumption of oxygen by *Melanoplus* eggs of different ages. They found that the Q_{10} of this process varied with the age of the eggs, but that dehydration caused no further change, even when the eggs had collapsed through loss of water. The dehydration was effected by concentrated Belar solutions, so that the conclusion to be drawn from their experiments is similar to that drawn below, from the experiments with strong salt solutions set out in section III above. Finally, Clark (1921) found that the Q_{10} of the frequency of heart beat in *Rana* was independent of pH above 15°C. At lower temperatures the rate of beat, and the Q_{10}, were higher at pH 8 than at pH 9.

Bělehrádek (1935)[1] believes that both the presence of electrolytes and change in the water content of protoplasm result in changes of protoplasmic viscosity, and thus in the temperature coefficients of all metabolic processes. This view has been criticized by Stiles (1930) on the grounds that viscosity changes need not be reflected in rates of metabolism. It is extremely unlikely that the viscosity of the protoplasm of *B. cereus* was unaffected either by change of pH over the range 6.0 to 7.5 or by addition of sodium chloride in concentrations up to 1.8 M within this pH range, especially in view of the marked diminution in the volume of the cells in salt solutions. Cells of *B. cereus* shrink to less than 80 per cent of their initial volume on suspension in 2 M sodium chloride (writer's unpublished observation). The viscosity of proteins is markedly reduced by the presence of salts (Kruyt and Lier, 1929), except near the isoelectric point where it is very susceptible to fluctuations of pH (Pauli and Matula, 1933) and it is probable that electrolytes bring about similar changes in protoplasm. Chambers and Reznikoff (1925–28) showed that sodium salts reduce the viscosity of protoplasm, and Jacobs (1922) and Prát (1926) have shown that small amounts of acids produce a similar effect. Thus adopting the view that a decrease in protoplasmic viscosity results in decreased temperature coefficients, one would expect salts or acidity to reduce them considerably. The evidence shows that this does not occur, so that it is improbable that the viscosity of the protoplasm determines the temperature coefficient of respiration in *B. cereus*.

The values of μ calculated from Figs. 1–3 approximate to the value 16,700 calories, which is believed by Crozier (1924–25) to characterize processes controlled by an iron dehydrogenation. It is not desirable to deduce the changes of μ with temperature from these figures, as there is no evidence to suggest that there are critical temperatures at which sudden changes occur, such as have been postulated by Crozier and Navez (1930–31). The values of μ calculated from Figs. 1–3 are averages over the range 13 to 27°C., and observations at lower temperatures were disregarded as being obviously inconsistent with these values. It is probable that in the present case a dehydrogenation is involved, for the inhibition of respiration in *B. cereus* by salts is due to inhibition of the dehydrogenases which limit respiration (Ingram, 1938, *cf.* also Quastel and Wooldridge, 1927). Further, when it is remembered that the increase in the temperature coefficient in dilute salt solutions is associated with an increase in the rate of metabolism, the master reaction concept may be seen to provide a reason-

[1] Bělehrádek, J., Temperature and living matter, Protoplasma Monograph No. 8, Berlin, Gebrüder Borntraeger, 1935, 69.

able explanation of the phenomena. The inhibition observed when salts are added is that of a limiting reaction, and this is likely to remain limiting if its rate is lowered. The constancy of the temperature coefficient during inhibition of respiration may thus be regarded as the result of the slowing of a reaction, already the slowest.

It might further be supposed that the alteration on stimulation could represent the beginning of a change-over to a new master reaction. Such a plan would be unsatisfactory from a quantitative point of view. Burton (1937) has demonstrated that very large changes in the relative rates of reactions are necessary before one can become "master" over others in the determination of a temperature coefficient. In the present experiments the temperature coefficient changed appreciably with an increase of less than 20 per cent in the rate of respiration.

The work described above was carried out as part of the program of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

V

SUMMARY

Measurements were made of the rate of consumption of oxygen by suspensions of *B. cereus*, in sodium chloride solutions of concentration up to 1.8 M and over a range of pH from 6.0 to 7.5. It was found:

1. That the temperature coefficient was independent of the presence of sodium chloride in concentrations between 0.2 and 1.8 M, although the rate of respiration was lowered considerably under these conditions.

2. That in the presence of concentrations of sodium chloride less than 0.2 M, the rate of respiration was increased, and so was the temperature coefficient.

3. That small changes in the temperature coefficient occurred when the pH was changed. The temperature coefficient was higher the higher the rate of respiration.

These data may be more readily interpreted by the hypothesis that the temperature coefficient is controlled by some master reaction, than by that which supposes that the temperature coefficient is determined by protoplasmic viscosity.

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INDEX TO AUTHORS

ANSON, M. L. The denaturation of proteins by synthetic detergents and bile salts, 239
—. The purification of cathepsin, 695
—. The reactions of denatured egg albumin with ferricyanide, 247
—. The reactions of iodine and iodoacetamide with native egg albumin, 321

BARNES, T. CUNLIFFE. Summation of electrical potential of living membranes (frog skin): a model of the electrical organ of fishes, 729
BLINKS, L. R. Bioelectric potentials in *Halicystis*. VIII. The effects of light, 495
— and NIELSEN, JOHN P. The cell sap of *Hydrodictyon*, 551
BRONFENBRENNER, J. See KALMANSON and BRONFENBRENNER, 203
BUCHWALD, CHARLES E. See WHELDEN, BUCHWALD, COOPER, and HASKINS, 391

CHASE, AURIN M., and SMITH, EMIL L. Regeneration of visual purple in solution, 21
CLOWES, G. H. A., and KRAHL, M. E. Studies on cell metabolism and cell division. III. Oxygen consumption and cell division of fertilized sea urchin eggs in the presence of respiratory inhibitors, 401
—. See KRAHL and CLOWES, 413
COLE, WILLIAM H. The composition of fluids and sera of some marine animals and of the sea water in which they live, 575
COMMONER, BARRY. See THIMANN and COMMONER, 333
COOPER, FRANKLIN S. See WHELDEN, BUCHWALD, COOPER, and HASKINS, 391
CROZIER, W. J. On the relation between birth weight and litter size, in mice, 309
— and HOLWAY, A. H. Theory and measurement of visual mechanisms. III. ΔI as a function of area, intensity, and wave length, for monocular and binocular stimulation, 101
— and WOLF, ERNST. The flicker response contour for the crayfish. I, 1
— and —. The flicker response contour for the frog, 229
— and —. The flicker response curve for *Fundulus*, 677
— and —. Reaction to visual flicker in the newt *Triturus*, 667
— and —. Temperature and critical illumination for reaction to flickering light. V. *Xiphophorus*, *Platypoecilius*, and their hybrids, 143
— and —. VI. Flash duration varied, 531

DAVIS, ALVA R. See WINNICK, DAVIS, and GREENBERG, 275, 289, 301
DELBRÜCK, M. Adsorption of bacteriophage under various physiological conditions of the host, 631
—. The growth of bacteriophage and lysis of the host, 643
DESREUX, VICTOR. See HERRIOTT, DESREUX, and NORTHROP, 439
DOUDOROFF, MICHAEL. Experiments on the adaptation of *Escherichia coli* to sodium chloride, 585

EVANS, HERBERT M. See LI, EVANS, and WONDER, 733
—. See LI, LYONS, and EVANS, 433

FRENCH, C. S. The pigment-protein compound in photosynthetic

bacteria. I. The extraction and properties of photosynthin, 469
FRENCH, C. S. The pigment-protein compound in photosynthetic bacteria. II. The absorption curves of photosynthin from several species of bacteria, 483

GORIN, MANUEL. See VELICK and GORIN, 753
GREENBERG, DAVID M. See WINNICK, DAVIS, and GREENBERG, 275, 289, 301
GUTTMAN, RITA. Stabilization of spider crab nerve membranes by alkaline earths, as manifested in resting potential measurements, 343

HADIDIAN, ZAREH, and HOAGLAND, HUDSON. Chemical pacemakers. Part I. Catalytic brain iron. Part II. Activation energies of chemical pacemakers, 81
HALPERIN, M. H. See MCFARLAND and HALPERIN, 613
HASKINS, CARYL P. See WHELDEN, BUCHWALD, COOPER, and HASKINS, 391
HECHT, SELIG, and PIRENNE, MAURICE HENRI. The sensibility of the nocturnal long eared owl in the spectrum, 709
HERRIOTT, ROGER M., DESREUX, VICTOR, and NORTHROP, JOHN H. Electrophoresis of pepsin, 439
HERSHEY, A. D. Factors limiting bacterial growth. VI. Equations describing the early periods of increase, 11
HILL, S. E. See OSTERHOUT and HILL, 743
HOAGLAND, HUDSON. See HADIDIAN and HOAGLAND, 81
HÖBER, RUDOLF. Studies concerning the nature of the secretory activity of the isolated Ringer-perfused frog liver. I. The differential secretion of pairs of dyestuffs, 185
— and MOORE, ELINOR. Studies concerning the nature of the secretory activity of the isolated Ringer-perfused frog liver. II. The inhibitory and the promoting influence of organic electrolytes and non-electrolytes upon the secretion of dyestuffs, 191
HOLWAY, A. H. See CROZIER and HOLWAY, 101

INGRAM, M. The influence of sodium chloride and temperature on the endogenous respiration of *Bacillus cereus*, 773
INMAN, O. L. Studies on the chlorophylls and photosynthesis of thermal algae from Yellowstone National Park, California, and Nevada, 661

JACQUES, A. G. The kinetics of penetration. XX. Effect of pH and of light on absorption in impaled *Halicystis*, 41
—. Separation of potassium isotopes in *Valonia* and *Nitella*, 741

KALMANSON, G., and BRONFENBRENNER, J. Studies on the purification of bacteriophage, 203
KRAHL, M. E., and CLOWES, G. H. A. Studies on cell metabolism and cell division. IV. Combined action of substituted phenols, cyanide, carbon monoxide, and other respiratory inhibitors on respiration and cell divsion, 413
—. See CLOWES and KRAHL, 401
KRUEGER, A. P., SCRIBNER, E. J., and MECRACKEN, T. The photodynamic inactivation of phage precursor by methylene blue, 705

LI, CHOH HAO, EVANS, HERBERT M., and WONDER, DONALD H. Electrophoretic homogeneity of pregnant mare serum gonadotrophin, 733
—, LYONS, WILLIAM R., and EVANS, HERBERT M. Studies on pituitary lactogenic hormone. I. Electrophoretic behavior, 433
LORING, HUBERT S. Solubility studies on purified tobacco mosaic virus, 719

LYDDANE, R. H., and STUHLMAN, OTTO, JR. The theoretical response of living cells to contact with solid bodies, 521

LYONS, WILLIAM R. See LI, LYONS, and EVANS, 433

MCFARLAND, R. A., and HALPERIN, M. H. The relation between foveal visual acuity and illumination under reduced oxygen tension, 613

MECRACKEN, T. See KRUEGER, SCRIBNER, and MECRACKEN, 705

MOORE, ELINOR. See HÖBER and MOORE, 191

MURRAY, J. W. See OSTERHOUT and MURRAY, 365

NIELSEN, JOHN P. See BLINKS and NIELSEN, 551

NORTHROP, JOHN H. Increase in bacteriophage and gelatinase concentration in cultures of *Bacillus megatherium*, 59

—. See HERRIOTT, DESREUX, and NORTHROP, 439

OSTERHOUT, W. J. V. Calculations of bioelectric potentials. V. Potentials in *Halicystis*, 53

—. VI. Some effects of guaiacol on *Nitella*, 171

—. Effects of guaiacol and hexylresorcinol in the presence of barium and calcium, 749

—. Effects of hexylresorcinol on *Nitella*, 569

—. Some chemical aspects of the potassium effect, 429

— and HILL, S. E. Action curves with single peaks in *Nitella* in relation to the movement of potassium, 743

— and MURRAY, J. W. Behavior of water in certain heterogeneous systems, 365

PETERS, ERICH, and SASLOW, GEORGE. Performance of the Hepp micro-osmometer, 177

PIRENNE, MAURICE HENRI. See HECHT and PIRENNE, 709

PRICE, JOHN W. Time-temperature relations in the incubation of the whitefish, *Coregonus clupeaformis* (Mitchill), 449

SASLOW, GEORGE. See PETERS and SASLOW, 177

SCRIBNER, E. J. See KRUEGER, SCRIBNER, and MECRACKEN, 705

SMITH, EMIL L. See CHASE and SMITH, 21

STUHLMAN, OTTO, JR. See LYDDANE and STUHLMAN, 521

SUBBAROW, Y., and TRAGER, W. The chemical nature of growth factors required by mosquito larvae. II. Pantothenic acid and vitamin B_6, 561

TEORELL, TORSTEN. On the permeability of the stomach mucosa for acids and some other substances, 263

THIMANN, KENNETH V., and COMMONER, BARRY. A differential volumeter for micro respiration measurements, 333

TRAGER, W. See SUBBAROW and TRAGER, 561

VELICK, SIDNEY, and GORIN, MANUEL. The electrical conductance of suspensions of ellipsoids and its relation to the study of avian erythrocytes, 753

WHELDEN, ROY M., BUCHWALD, CHARLES E., COOPER, FRANKLIN S., and HASKINS, CARYL P. Electron bombardment of biological materials. II. The rate of death of fungus spores bombarded in vacuum with cathode ray beams from 4 kv.–15 kv., 391

WILKERSON, VERNON A. The chemistry of human skin. IV. The electrokinetic effect of various ions upon suspended particles of stratum corneum, 165

WINNICK, THEODORE, DAVIS, ALVA R., and GREENBERG, DAVID M. Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. I. Chemical properties, activation-inhibition, pH-activity, and temperature-activity curves, 275

—, —, and —. II. Kinetics of protein digestion by asclepain, 289

WINNICK, THEODORE, DAVIS, ALVA R., and GREENBERG, DAVID M. Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. III. Kinetics of the heat inactivation of papain, bromelin, and asclepain, 301

WOLF, ERNST. See CROZIER and WOLF, 1, 143, 229, 531, 667, 677

WONDER, DONALD H. See LI, EVANS, and WONDER, 733

INDEX TO SUBJECTS

ABSORPTION curves of photosynthin, pigment-protein compound, from photosynthetic bacteria, 483
— in impaled *Halicystis*, effect of hydrogen ion concentration and of light, 41
Acid, pantothenic, and vitamin B_6, chemical nature of growth factors required by mosquito larvae, 561
Acids and other substances, permeability of stomach mucosa for, 263
Adsorption of bacteriophage under various physiological conditions of host, 631
Albumin, egg, denatured, reactions with ferricyanide, 247
—, egg, native, reactions of iodine and iodoacetamide with, 321
Algae, thermal, from Yellowstone National Park, California, and Nevada, chlorophylls and photosynthesis, 661
Alkaline earths, stabilization of spider crab nerve membranes by, as manifested in resting potential measurements, 343
Animals, marine, composition of fluids and sera, and of sea water in which they live, 575
Asclepain, kinetics of protein digestion by, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 289
—, papain, and bromelin, kinetics of heat inactivation, comparison of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with, 301
Asclepias speciosa Torr., physicochemical properties of proteolytic enzyme from latex, comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
Asclepias speciosa Torr., physicochemical properties of proteolytic enzyme from latex, comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
— *speciosa* Torr., physicochemical properties of proteolytic enzyme from latex, comparisons with other proteases, kinetics of protein digestion by asclepain, 289
Avian erythrocytes, relation of electrical conductance of ellipsoid suspensions, 753

BACILLUS *cereus*, endogenous respiration, influence of sodium chloride and temperature, 773
— *megatherium* cultures, increase in bacteriophage and gelatinase concentration in, 59
Bacteria, photosynthetic, containing pigment-protein compound, photosynthin, absorption curves, 483
—, photosynthetic, containing pigment-protein compound, photosynthin, extraction and properties, 469
Bacterial growth, factors limiting, equations describing early periods of increase, 11
Bacteriophage, adsorption under various physiological conditions of host, 631
— and gelatinase concentration, increase in cultures of *Bacillus megatherium*, 59
—, growth, and lysis of host, 643
— precursor, photodynamic inactivation by methylene blue, 705
—, purification, 203

Barium and calcium, effects of guaiacol and hexylresorcinol in presence of, 749
Bile salts and synthetic detergents, denaturation of proteins by, 239
Bioelectric potentials, calculations, effects of guaiacol on *Nitella*, 171
— potentials in *Halicystis*, calculations, 53
— potentials in *Halicystis*, effects of light, 495
Biological materials, electron bombardment, rate of death of fungus spores bombarded in vacuum with cathode ray beams from 4 to 15 kilovolts, 391
Birth weight and litter size, relation in mice, 309
Brain iron, catalytic, and activation energies of chemical pacemakers, 81
Bromelin, papain, and asclepain, kinetics of heat inactivation, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with, 301

CALCIUM and barium, effects of guaiacol and hexylresorcinol in presence of, 749
California, Nevada, and Yellowstone National Park, chlorophylls and photosynthesis of thermal algae from, 661
Carbon monoxide, substituted phenols, cyanide, and respiratory inhibitors, combined action on respiration and cell division, 413
Catalytic brain iron, and activation energies of chemical pacemakers, 81
Cathepsin, purification, 695
Cathode ray beams from 4 to 15 kilovolts, death rate of fungus spores bombarded in vacuum with, 391
Cell metabolism and division, 401, 413
— sap of *Hydrodictyon*, 551
Cells, living, theoretical response to contact with solid bodies, 521
Chemical aspects of potassium effect, 429
— nature of growth factors required by mosquito larvae, pantothenic acid and vitamin B_6, 561
— pacemakers, catalytic brain iron, and activation energies of, 81
—, physico-, properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
—, physico-, properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, kinetics of protein digestion by asclepain, 289
— properties, activation-inhibition, and influence of hydrogen ion concentration and temperature on proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, 275
Chemistry of human skin, electrokinetic effect of ions upon suspended particles of stratum corneum, 165
Chlorophylls and photosynthesis of thermal algae from Yellowstone National Park, California, and Nevada, 661
Conductance, electrical, of suspensions of ellipsoids, relation to study of avian erythrocytes, 753
Coregonus clupeaformis Mitchill, time-temperature relations in incubation, 449
Crab, spider, nerve membranes, stabilization by alkaline earths, as manifested in resting potential measurements, 343
Crayfish, flicker response contour, 1
Cultivation, *Bacillus megatherium*, increase in bacteriophage and gelatinase concentration, 59
Cyanide, substituted phenols, carbon monoxide, and respiratory inhibitors, combined action on respiration and cell division, 413

DEATH rate of fungus spores bombarded in vacuum with cathode ray beams from 4 to 15 kilovolts, 391
Detergents, synthetic, and bile salts, denaturation of proteins by, 239
Digestion, protein, by asclepain, kinetics, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 289
Dyestuffs, pairs, differential secretion by isolated Ringer-perfused frog liver, 185
—, secretion by isolated Ringer-perfused frog liver, inhibitory and promoting influence of organic electrolytes and non-electrolytes, 191

EGG albumin, denatured, reactions with ferricyanide, 247
— albumin, native, reactions of iodine and iodoacetamide with, 321
Eggs, sea urchin, fertilized, oxygen consumption and cell division in presence of respiratory inhibitors, 401
Electric potential of living membranes, frog skin, 729
— potentials in *Halicystis*, calculations, 53
— potentials in *Halicystis*, effects of light, 495
— potentials in *Nitella*, calculations, effects of guaiacol, 171
Electrical conductance of suspensions of ellipsoids and relation to study of avian erythrocytes, 753
Electrokinetic effect of ions upon suspended particles of stratum corneum, 165
Electrolytes and non-electrolytes, organic, inhibitory and promoting influence upon secretion of dyestuffs by isolated Ringer-perfused frog liver, 191
Electron bombardment of biological materials, rate of death of fungus spores bombarded in vacuum with cathode ray beams from 4 to 15 kilovolts, 391
Electrophoresis of pepsin, 439
Electrophoretic behavior of pituitary lactogenic hormone, 433
— homogeneity of pregnant mare serum gonadotrophin, 733
Ellipsoids, suspensions, electrical conductance, relation to study of avian erythrocytes, 753
Enzyme, proteolytic, from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
—, proteolytic, from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
—, proteolytic, from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, kinetics of protein digestion by asclepain, 289
Erythrocytes, avian, relation of electrical conductance of ellipsoid suspensions, 753
Escherichia coli, adaptation to sodium chloride, 585
Eye, monocular and binocular stimulation, ΔI as function of area, intensity, and wave length for, 101

FERRICYANIDE, reactions of denatured egg albumin with, 247
Fertilized sea urchin eggs, oxygen consumption and cell division, in presence of respiratory inhibitors, 401
Fish, *Coregonus clupeaformis* Mitchill, time-temperature relations in incubation, 449
Fishes, electrical organ, model, 729
Fluids and sera of marine animals, and sea water in which they live, composition, 575
Foveal visual acuity, relation to illumination under reduced oxygen tension, 613
Frog, flicker response contour, 229

Frog skin, living membranes, electrical potential, 729
Fundulus, flicker response curve, 677
Fungus spores bombarded in vacuum with cathode ray beams from 4 to 15 kilovolts, death rate, 391

GELATINASE and bacteriophage concentration, increase in cultures of *Bacillus megatherium*, 59
Gonadotrophin, pregnant mare serum, electrophoretic homogeneity, 733
Growth, bacterial, factors limiting, equations describing early periods of increase, 11
— factors required by mosquito larvae, chemical nature, pantothenic acid and vitamin B_6, 561
— of bacteriophage and lysis of host, 643
Guaiacol, effects on *Nitella*, calculations of bioelectric potentials, 171
— and hexylresorcinol, effects in presence of barium and calcium, 749

HALICYSTIS, bioelectric potentials, calculations, 53
—, bioelectric potentials, effects of light, 495
—, impaled, effect of hydrogen ion concentration and of light on absorption in, 41
Heat inactivation of papain, bromelin, and asclepain, kinetics, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 301
Hexylresorcinol, effects on *Nitella*, 569
— and guaiacol, effects in presence of barium and calcium, 749
Hormone, pituitary, lactogenic, electrophoretic behavior, 433
Horse, pregnant, serum gonadotrophin, electrophoretic homogeneity, 733
Hybrids of *Xiphophorus* and *Platypoecilius*, temperature and critical illumination for reaction to flickering light, 143
Hydrodictyon, cell sap, 551
Hydrogen ion concentration, effect on absorption in impaled *Halicystis*, 41
— ion concentration, influence on proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, 275

IODINE and iodoacetamide, reactions with native egg albumin, 321
Iodoacetamide and iodine, reactions with native egg albumin, 321
Ions, electrokinetic effect upon suspended particles of human stratum corneum, 165
Iron, brain, catalytic, and activation energies of chemical pacemakers, 81
Isotopes, potassium, separation in *Valonia* and *Nitella*, 741

KINETICS of heat inactivation of papain, bromelin, and asclepain, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 301
— of penetration, effect of hydrogen ion concentration and of light on absorption in impaled *Halicystis*, 41
— of protein digestion by asclepain, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 289

LACTOGENIC hormone, pituitary, electrophoretic behavior, 433
Larvae, mosquito, chemical nature of growth factors required, pantothenic acid and vitamin B_6, 561
Latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties of proteolytic enzyme from, comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
— of milkweed, *Asclepias speciosa* Torr., physicochemical properties of pro-

teolytic enzyme from, comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
Latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties of proteolytic enzyme from, comparisons with other proteases, kinetics of protein digestion by asclepain, 289
Light, effect on absorption in impaled *Halicystis*, 41
—, effects on bioelectric potentials in *Halicystis*, 495
—, flicker response contour for frog, 229
—, flickering, temperature and critical illumination for reaction of *Xiphophorus*, *Platypoecilius*, and their hybrids, 143
—, flickering, temperature and critical illumination for reaction to, flash duration varied, 531
Litter size and birth weight in mice, relation between, 309
Liver, frog, Ringer-perfused, isolated, differential secretion of pairs of dyestuffs, 185
—, frog, Ringer-perfused, isolated, inhibitory and promoting influence of organic electrolytes and non-electrolytes upon secretion of dyestuffs, 191
Lysis of host, and growth of bacteriophage, 643

MAN, skin, chemistry, electrokinetic effect of ions upon suspended particles of stratum corneum, 165
Membranes, living, frog skin, electrical potential, 729
—, nerve, spider crab, stabilization by alkaline earths, as manifested in resting potential measurements, 343
Metabolism, cell, and cell division, 401, 413
Methylene blue, photodynamic inactivation of phage precursor by, 705
Micro osmometer, Hepp, performance, 177
Micro respiration measurements, differential volumeter, 333
Milkweed, *Asclepias speciosa* Torr., latex, physicochemical properties of proteolytic enzyme from, comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
—, *Asclepias speciosa* Torr., latex, physicochemical properties of proteolytic enzyme from, comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
—, *Asclepias speciosa* Torr., latex, physicochemical properties of proteolytic enzyme from, comparisons with other proteases, kinetics of protein digestion by asclepain, 289
Mosaic virus, tobacco, purified, solubility, 719
Mosquito larvae, chemical nature of growth factors required, pantothenic acid and vitamin B_6, 561
Mucosa, stomach, permeability for acids and other substances, 263

NERVE membranes, spider crab, stabilization by alkaline earths, as manifested in resting potential measurements, 343
Nevada, California, and Yellowstone National Park, chlorophylls and photosynthesis of thermal algae from, 661
Newt, *Triturus*, reaction to visual flicker, 667
Nitella, action curves with single peaks, in relation to movement of potassium, 743
—, effects of guaiacol, calculations of bioelectric potentials, 171
—, effects of hexylresorcinol, 569
— and *Valonia*, separation of potassium isotopes in, 741

ORGAN, electrical, fishes, model, 729
Organic electrolytes and non-electrolytes, inhibitory and promoting influence upon secretion of dyestuffs by isolated Ringer-perfused frog liver, 191

Osmometer, micro, Hepp, performance, 177
Owl, nocturnal, long eared, sensibility in spectrum, 709
Oxygen consumption and cell division of fertilized sea urchin eggs in presence of respiratory inhibitors, 401
— tension, reduced, relation between foveal visual acuity and illumination, 613

PANTOTHENIC acid and vitamin B_6, chemical nature of growth factors required by mosquito larvae, 561
Papain, bromelin, and asclepain, kinetics of heat inactivation, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with, 301
Penetration, kinetics, effect of hydrogen ion concentration and of light on absorption in impaled *Halicystis*, 41
Pepsin, electrophoresis, 439
Perfusion, Ringer, nature of secretory activity of isolated frog liver, 185, 191
Permeability of stomach mucosa for acids and other substances, 263
Phenols, substituted, cyanide, carbon monoxide, and respiratory inhibitors, combined action on respiration and cell division, 413
Photodynamic inactivation of phage precursor by methylene blue, 705
Photosynthesis and chlorophylls of thermal algae from Yellowstone National Park, California, and Nevada, 661
Photosynthetic bacteria containing pigment-protein compound, photosynthin, absorption curves, 483
— bacteria containing pigment-protein compound, photosynthin, extraction and properties, 469
Photosynthin, pigment-protein compound in photosynthetic bacteria, absorption curves, 483
Photosynthin, pigment-protein compound in photosynthetic bacteria, extraction and properties, 469
Physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
— properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
— properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, kinetics of protein digestion by asclepain, 289
Physiological conditions of host, influence on adsorption of bacteriophage, 631
Pigment-protein compound, photosynthin, in photosynthetic bacteria, absorption curves, 483
— compound, photosynthin, in photosynthetic bacteria, extraction and properties, 469
Pituitary lactogenic hormone, electrophoretic behavior, 433
Platypoecilius, *Xiphophorus*, and hybrids, reaction to flickering light, temperature and critical illumination for, 143
Potassium effect, chemical aspects, 429
— isotopes in *Valonia* and *Nitella*, separation, 741
—, movement, relation of action curves with single peaks in *Nitella*, 743
Potential, electrical, of living membranes, frog skin, 729
— measurements, resting, stabilization of spider crab nerve membranes by alkaline earths, manifested in, 343
Potentials, bioelectric, calculations, effect of guaiacol on *Nitella*, 171

Potentials, bioelectric, in *Halicystis*, calculations, 53
—, bioelectric, in *Halicystis*, effects of light, 495
Pregnant mare serum gonadotrophin, electrophoretic homogeneity, 733
Proteases, and proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons of physicochemical properties, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
—, and proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons of physicochemical properties, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
—, and proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons of physicochemical properties, kinetics of protein digestion by asclepain, 289
Protein digestion by asclepain, kinetics, comparisons of physicochemical properties of proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., with other proteases, 289
Protein-pigment compound, photosynthin, in photosynthetic bacteria, absorption curves, 483
— compound, photosynthin, in photosynthetic bacteria, extraction and properties, 469
Proteins, denaturation by synthetic detergents and bile salts, 239
Proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, activation-inhibition and influence of hydrogen ion concentration and temperature, 275
— enzyme from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, kinetics of heat inactivation of papain, bromelin, and asclepain, 301
— enzyme from latex of milkweed, *Asclepias speciosa* Torr., physicochemical properties, comparisons with other proteases, kinetics of protein digestion by asclepain, 289

RESPIRATION, endogenous, of *Bacillus cereus*, influence of sodium chloride and temperature, 773
—, micro, measurements, differential volumeter, 333
Respiratory inhibitors, combined action of substituted phenols, cyanide, and carbon monoxide on respiration and cell division, 413
— inhibitors, oxygen consumption and cell division of fertilized sea urchin eggs in presence of, 401
Ringer-perfused frog liver, isolated, differential secretion of pairs of dyestuffs, 185
— frog liver, isolated, inhibitory and promoting influence of organic electrolytes and non-electrolytes upon secretion of dyestuffs, 191

SALTS, bile, and synthetic detergents, denaturation of proteins by, 239
Sea urchin eggs, fertilized, oxygen consumption and cell division, in presence of respiratory inhibitors, 401
Secretory activity of isolated Ringer-perfused frog liver, 185, 191
Sensibility of nocturnal long eared owl in spectrum, 709
Sera and fluids of marine animals, and sea water in which they live, composition, 575
Serum, pregnant mare, gonadotrophin, electrophoretic homogeneity, 733
Skin, frog, living membranes, electrical potential, 729
—, human, chemistry, electrokinetic effect of ions upon suspended particles of stratum corneum, 165
Sodium chloride, adaptation of *Escherichia coli* to, 585
— chloride and temperature, influence on endogenous respiration of *Bacillus cereus*, 773
Solid bodies, theoretical response of living cells to contact with, 521

Solubility studies on purified tobacco mosaic virus, 719
Spectrum, sensibility of nocturnal long eared owl in, 709
Spider crab nerve membranes, stabilization by alkaline earths, as manifested in resting potential measurements, 343
Spores, fungus, bombarded in vacuum with cathode ray beams from 4 to 15 kilovolts, death rate, 391
Springs, hot, thermal algae from Yellowstone National Park, California, and Nevada, chlorophylls and photosynthesis, 661
Stimulation, monocular and binocular, ΔI as function of area, intensity, and wave length for, 101
Stomach mucosa, permeability for acids and other substances, 263
Stratum corneum, human, suspended particles, electrokinetic effect of ions upon, 165

TEMPERATURE and critical illumination for reaction of *Xiphophorus*, *Platypoecilius*, and their hybrids to flickering light, 143
— and critical illumination for reaction to flickering light, flash duration varied, 531
—, influence on proteolytic enzyme from latex of milkweed, *Asclepias speciosa* Torr., comparisons with other proteases, 275
— and sodium chloride, influence on endogenous respiration of *Bacillus cereus*, 773
Temperature-time relations in incubation of whitefish, *Coregonus clupeaformis* Mitchill, 449
Tension, oxygen, reduced, relation between foveal visual acuity and illumination, 613
Tobacco mosaic virus, purified, solubility, 719
Triturus, reaction to visual flicker, 667

VACUUM, death rate of fungus spores bombarded in, with cathode ray beams from 4 to 15 kilovolts, 391
Valonia and *Nitella*, separation of potassium isotopes in, 741
Virus, tobacco mosaic, purified, solubility, 719
Vision, acuity, foveal, relation to illumination under reduced oxygen tension, 613
—, mechanisms, theory and measurement, ΔI as function of area, intensity, and wave length, for monocular and binocular stimulation, 101
—, reaction to flicker in newt *Triturus*, 667
—, regeneration of visual purple in solution, 21
Vitamin B_6 and pantothenic acid, chemical nature of growth factors required by mosquito larvae, 561
Volumeter, differential, for micro respiration measurements, 333

WATER, behavior in heterogeneous systems, 365
—, sea, in which marine animals live, composition, and composition of their fluids and sera, 575
Weight, birth, and litter size, relation in mice, 309
Whitefish, *Coregonus clupeaformis* Mitchill, time-temperature relations in incubation, 449

XIPHOPHORUS, *Platypoecilius*, and their hybrids, reaction to flickering light, temperature and critical illumination for, 143

YELLOWSTONE National Park, California, and Nevada, chlorophylls and photosynthesis of thermal algae from, 661

I.A R.I. 75

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Date of Issue	Date of Issue	Date of Issue
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